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**Genetic investigation of $\alpha 4$ -containing
GABA_A receptors' different roles in
alcohol consumption and conditioned
behaviours influenced by cocaine**

Jonathan Robertson

DPhil in Psychology

University of Sussex

November 2017

I hereby declare that this thesis has not been and will not be submitted in whole or in part to another University for the reward of any other degree.

Signature

University of Sussex

Jonathan Robertson

Genetic investigation of $\alpha 4$ -containing GABA_A receptors' different roles in alcohol consumption and conditioned behaviours influenced by cocaine

The GABA_A $\alpha 4$ -subunit is found co-assembled with δ subunits in extrasynaptic GABA_A receptors ($\alpha 4$ -GABA_ARs). Within the striatum $\alpha 4$ -GABA_ARs are most highly expressed in the Nucleus Accumbens (NAc) where they mediate tonic inhibition thought to control the excitability of accumbal medium spiny neurons (MSNs). Experiments presented in this thesis use genetic techniques in mice to investigate the role of $\alpha 4$ -GABA_ARs in modulating binge-like ethanol consumption and the potentiation of locomotor behaviours by cocaine. We have generated several transgenic mouse lines in which the *Gabra4* gene, encoding the $\alpha 4$ subunit, has been deleted either constitutively or within specific neural populations expressing D1 or D2 type dopamine receptors via cre/loxp recombination. Using quantitative rt-PCR and in-situ-hybridisation methods to compare *Gabra4* mRNA levels in brain sections from each genotype we confirmed that the $\alpha 4$ subunit was deleted either globally or in the expected cell type within conditional knockouts. We also generated an Adeno Associated Virus (AAV) carrying Cre-recombinase to knockdown $\alpha 4$ locally by infusing it into specific brain regions of 'floxed'- $\alpha 4$ mice.

Deletion of the $\alpha 4$ subunit in mice significantly reduced alcohol consumption in a pre-clinical model of binge-drinking, known as drinking in the dark (DID). Moreover, targeted deletion of *Gabra4* in the NAc was sufficient to mediate this effect. We did not observe any effects on alcohol consumption in mice where $\alpha 4$ was deleted conditionally in D1 or D2 type neurons. This data indicates that $\alpha 4$ -GABA_ARs in the NAc are an important mediator of alcohol consumption.

Deletion of GABA_A $\alpha 4$ -subunits from dopamine D1-expressing neurons facilitated cocaine's ability to potentiate locomotor activity and operant responding for natural rewards. Deletion of GABA_A $\alpha 4$ -subunits from dopamine D2-expressing neurons had no such effects. Deletion of GABA_A $\alpha 4$ -subunits from dopamine D1-expressing neurons also accelerated the acquisition of behavioural sensitisation to cocaine. This effect was associated with increased cFos expression in the NAc core following acute cocaine, whilst in cocaine-sensitised mice it was associated with increased cFos in both the NAc Core and Shell. A similar altered pattern of cFos expression was observed in mice with a global knockout of $\alpha 4$ subunits however they showed no behavioural effects. This may imply that a balance of $\alpha 4$ -GABA_AR-mediated inhibition in D1 and D2 neurons is required for normal behavioural sensitisation to cocaine. The data presented within this thesis indicate that $\alpha 4$ -GABA_AR-mediated inhibition of D1- and D2-expressing neurons plays an important physiological role in controlling behavioural responses to cocaine.

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List of Abbreviations

$\alpha 2$ -GABA _A R	=	$\alpha 2$ -containing GABA _A receptor
$\alpha 4$ -GABA _A R	=	$\alpha 4$ -containing GABA _A receptor
ACh	=	Acetylcholine
AMG	=	Amygdala
AMPA	=	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BAC	=	Bacterial Artificial Chromosome
BEC	=	Blood Ethanol Content
BDNF	=	Brain Derived Neutrophic Factor
BDZ	=	Benzodiazepine
BG	=	Basal Ganglia
BLA	=	Basolateral Amygdala
CDP	=	Chlordiazepoxide
CeA	=	Central Nucleus of the Amygdala
ChR	=	Channelrhodopsin
Cl ⁻	=	Chloride
CNS	=	Central Nervous System
CPA	=	Conditioned Place Avoidance
CPP	=	Conditioned Place Preference
CRf	=	Conditioned Reinforcement
CS	=	Conditioned Stimulus
DARPP-32	=	Dopamine- & cAMP-regulated neuronal phosphoprotein
DREADD	=	Designer Receptor Exclusively Activated Designer Drug

eGFP	=	Enhanced Green Fluorescent Protein
EPSP	=	Excitatory Postsynaptic Potential
GABA	=	γ -aminobutyric acid
GABA _A R	=	GABA _A receptor
GFP	=	Green Fluorescent Protein
GPe	=	Globus Pallidus externa
GPI	=	Globus Pallidus interna
GVG	=	Gamma-Vinyl GABA
HIP	=	Hippocampus
KO	=	Knockout
LTP	=	Long-Term Potentiation
mPFC	=	Medial Prefrontal Cortex
MSN	=	Medium Spiny Neuron
NAc	=	Nucleus Accumbens
NL2	=	Neurologin2
NMDA	=	N-methyl-D-aspartic acid
NpHR	=	Halorhodopsin
PFC	=	Prefrontal Cortex
PIT	=	Pavlovian to Instrumental Transfer
PPTg	=	Pedunculopontine Tegmental Nucleus
qRT-PCR	=	(Quantitative) Reverse Transcriptase Polymerase Chain Reaction
Ro 15-4513	=	Ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-1,4-benzodiazepine-3-carboxylate
SNC	=	Substantia Nigra pars compacta

SNr	=	Substantia Nigra pars reticulate
SNP	=	Single Nucleotide Polymorphism
STN	=	Subthalamic Nucleus
THIP	=	4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol
TM	=	Transmembrane
VI	=	Variable Interval
VP	=	Ventral Pallidum
VTA	=	Ventral Tegmental Area
WT	=	Wildtype

Chapter 1

General Introduction

1.1. Drug and alcohol abuse/addiction

The world health organisation (WHO) estimates that two hundred fifty million people used illicit drugs at least once in 2015, of which about 29.5 million of those drug users, or 0.6% of the global adult population, suffer from drug use disorders (WHO, 2017). Within the UK alone approximately 8.5% of adults used an illicit drug in 2015/16 (UK Home Office, 2017). Of these users, ~10-13% continue to be problem users with drug dependence and/or use disorders.

Cocaine use appears to be increasing in the two largest markets, North America and Europe, and the global disease burden attributed to cocaine use disorders increased by ~37% from 2005 to 2015 (WHO, 2017). During 2013/14, an estimated 2.4% of adults, aged 16-59 in England and Wales, used powder cocaine; making it the second most commonly used illegal drug (UK Advisory Council on the Misuse of Drugs, 2015).

Globally harmful use of alcohol has been estimated to cause 5.9% of annual deaths and account for 5.1% of the global health burden (WHO, 2017). In particular 'binge drinking', a pattern in which blood alcohol levels are raised to 0.08 grams alcohol per decilitre blood (0.8mg/ml), is associated with a large portion of alcohol related deaths, diseases and social harms (NIAAA, 2004). In the UK 58% of the population (28.9 million people) drink alcohol regularly, of which 26.8% of binge-drink on their heaviest dinking days (UK Office for National Statistics, 2016). During 2015/16, in England alone, there were 333 thousand estimated admissions where an alcohol-related disease, injury or condition was the primary diagnosis or there was an alcohol-related external cause.

The majority of treatments available for addiction are psychosocial interventions and behavioral therapies. The US National Institute on Drug Abuse (NIDA) estimate in developed countries rates of relapse during treatment are around 40-60% (NIDA, 2014). There are currently very few pharmaceutical treatments

available for addiction and they are not commonly used. Most currently used medications either act to replace the abused drug (e.g. methadone and nicotine supplements) or to block or make uncomfortable the effects of the drug (e.g. naltrexone and disulfiram) (NIDA, 2012). There is therefore a need for pre-clinical research into the cellular, molecular, genetic and behavioral etiology of addiction to identify targets for therapeutic treatments.

1.2. Brain regions involved in addiction

1.2.1. The Basal Ganglia

The basal ganglia (BG) are a series of interconnected subcortical nuclei primarily responsible for motor control as well as wider roles in motor learning, executive function and emotions. BG dysfunction therefore underlies a multitude of neuropathologies (Alexander and Crutcher, 1990; Cohen and Frank, 2009). The BG includes the striatum, globus pallidus externa (GPe) and interna (GPi), substantia nigra pars compacta (SNc) and pars reticulata (SNr), and subthalamic nucleus (STN).

The BG and related nuclei can be broadly categorized into three groups. Firstly, input nuclei receive incoming information from various sources, mainly the cortex, thalamus, VTA and Substantia Nigra, and consist of the Caudate, Putamen and Nucleus Accumbens (NAc). Output nuclei send basal ganglia information to the thalamus and consist of the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr). Finally, intrinsic nuclei are located between the input and output nuclei in the relay of information and include the GPe, STN and SNc.

Regions within the BG are anatomically linked to the cerebral cortex and thalamo-cortical motor system via a series of parallel cortico-basal ganglia-thalamo-cortical 'loops' which are largely structurally and functionally distinct (Alexander and Crutcher, 1990; Haber, 2003). Specific regions of the cortex send excitatory glutamatergic projections to the input structures of the BG, following which BG output nuclei exert a tonic GABA-mediated inhibitory control over their target nuclei in the thalamus. The thalamus then sends excitatory glutamatergic projections back to the cortex, thus completing the 'loop'. Via this structure cortical afferent activity is modulated by the basal ganglia, which

subsequently sends back a signal to the cortex to facilitate (or inhibit) motor activity (Mink, 1996; Nicola, 2007).

Classically, BG 'loops' were classified according to the presumed role of their primary cortical projection areas; motor, oculomotor, limbic, associative, and orbitofrontal circuits (Alexander and Crutcher, 1990; Mogenson and Yang, 1991; DeLong and Wichmann, 2007).

1.2.2. The direct and indirect pathways

Between the cortex and thalamus neuronal afferents are modulated by three distinct relay circuits known as the 'direct', 'indirect' and 'hyperdirect' pathways. The direct pathway originates in striatonigral neurons which form monosynaptic inhibitory connections with SNr/GPi neurons, suppressing inhibition of the thalamus, and ultimately disinhibiting selected behaviours (Vincent *et al.*, 1982; Christensson-Nylander *et al.*, 1986; Chevalier and Deniau, 1990). The indirect pathway originates in striatopallidal neurons which project to the GPe and onto the SNr/GPi complex via a polysynaptic disinhibitory connection, and an indirect GPe-STN-GPi connection, ultimately inhibiting the thalamus and suppressing selected behaviours (Beckstead and Kersey, 1985; Gerfen *et al.*, 1990; Albin, Young and Penney, 1995; Cohen and Frank, 2009).

Additionally the cortico-subthalamo-pallidal "hyperdirect" pathway was discovered, in which cortical afferents bypasses the striatum altogether, projecting directly to the STN (Nambu *et al.*, 2000). The STN sends diffuse excitatory projections to many GPI neurons, producing a global, rather than selective, suppression of responses (Frank, 2006; Cohen and Frank, 2009). This third pathway has been proposed to mediate premature response inhibition, and termination of initiated behaviours (Aron and Poldrack, 2006).

1.2.3. The Striatum

1.2.3.1. Architecture of the Striatum

The largest structure in the BG is the Striatum which comprises of the Caudate, Putamen, Nucleus Accumbens (NAc) and olfactory tubercle. The striatum serves as the primary afferent structure to the rest of the BG, precisely modulating neuronal excitability in the BG nuclei to mediate action-selection.

The vast majority (~95%) of neurons within the striatum are GABAergic projection medium spiny neurons (MSNs) (Wilson, 1993) so named due to their medium size and extensive dendritic trees (Kemp and Powell, 1971). MSNs receive glutamatergic inputs from the cortex, ventral hippocampus, amygdala and thalamus which synapse at their 'spines', and midbrain dopaminergic projections which synapse at the dendrites and 'spine necks' (Smith *et al.*, 1994).

MSNs of the direct and indirect pathway have different molecular profiles so they were initially identified as distinct subtypes using their releasable neuropeptides as cell-type specific markers (Gerfen and Scott Young, 1988; Le Moine and Bloch, 1995). Immunohistochemistry identified populations of striatal neurons expressing the neuropeptides dynorphin and substance P and others expressing enkephalin (Beckstead and Kersey, 1985; Christensson-Nylander *et al.*, 1986). Subsequent experiments in 6-hydroxydopamine (6-OHDA) lesioned mice used D1 and D2 specific agonists (SKF-38393 and quinpirole) in combination with retrograde tracing and mRNA profiling to demonstrate that dynorphin/substance P or enkephalin expression in MSNs were differentially associated with D1 or D2 dopamine receptor (D1R or D2R) expression respectively (Gerfen *et al.*, 1990).

Dopamine exerts a dual effect on MSNs, inhibiting striatal D2R-containing neurons and exciting striatal neurons that express D1Rs through L-type calcium channels (Onn, West and Grace, 2000). Further studies confirmed that D1Rs and D2Rs are expressed in distinct MSN subtypes (Le Moine & Bloch 1995) which are segregated into discrete pathways. Classically D1R-containing (D1) neurons are the origin of the 'direct pathway' and whilst D2R-containing (D2) neurons form part of the 'indirect' pathway. A large body of research has elucidated distinct and often opposing functions of these two populations, sometimes referred to as the go/no-go pathways due to their roles in action initiation/inhibition (Surmeier 2013).

1.2.3.2. Genetic Dissection of D1 and D2 pathways

Using recombinant DNA techniques genetic constructs can be placed under the control of various cis-regulatory elements. These can encode molecules which allow easy identification of the cells in which they are expressed, i.e. 'reporter

genes' (Forss-Petter *et al.*, 1990). This type of labelling is more reliable than immunohistochemistry which requires markers that were based on correlative data (Gerfen *et al.*, 1990) rather than being genetically hard-coded. The discovery and development of green fluorescent protein (GFP) as a marker for gene expression (Chalfie *et al.*, 1994) has rapidly become a staple technique for mapping neural populations as it allows stable reporter expression with minimal perturbation to the cell. Constructs encoding several GFP variants with different optical properties (collectively named XFP) were inserted under different promoters to allow imaging of neuronal subsets based on their expression patterns (Feng *et al.*, 2000). In a refinement of this gene-trapping method GFP was expressed under the control of regulatory elements which had already been defined as specific to certain cell types. This was achieved using Bacterial Artificial Chromosomes (BACs) as vectors for these reporters, allowing large regulatory sequences and buffering transgenes from local regulation (Gong *et al.*, 2003). This included 20 lines which drove expression within the striatum, of particular importance GFP was expressed under the *Drd1* or *Drd2* dopamine receptor promoters. By generating a BAC transgenic line expressing a different fluorophore (tdTomato) under the *Drd1* promoter and crossing it with one expressing GFP under the *Drd2* promoter it was possible to definitively dissociate D1 and D2 MSNs as only ~1% of neurons co-expressed both D1 and D2 receptors (Shuen *et al.*, 2008).

The bacteriophage P1 recombinase 'Cre' can be used to mediate recombination between short sequences called 'loxP' sites (Hoess and Abremski, 1985) and a Cre-lox recombination system has been developed for use in mammals (Sauer and Henderson, 1988). Transgenic mice expressing Cre can be crossed with mice carrying a sequence which has been flanked by loxP sites via homologous recombination ('floxed') which results in deletion of that intervening sequence in the offspring (Lakso *et al.*, 1992). This system is used in mice carrying a 'floxed' gene of interest so that it will be knocked out in a compartmentalised, cell-specific manner which is determined by the promoter under which Cre is regulated (Gu *et al.*, 1994). As previously with GFP reporters a library of BAC-Cre mice have been generated where Cre is expressed in genetically defined neural subtypes in over 250 driver lines (Gong *et al.*, 2007). Again, this includes expression of Cre under the *Drd1* or *Drd2* dopamine receptor promoters. This

technique has been used to conditionally delete genes in D1 or D2 expressing neurons with different behavioural consequences. Conditional knock-out of the BDNF receptor Trkb in D1 or D2 neurons either increased or decreased cocaine conditioned place preference (CPP) respectively. Similarly, conditional deletion of DARP-32, a MSN marker and signalling molecule, in D1 MSNs resulted in hypoactivity and reduced psychostimulant response whilst in D2 neurons deletion of DARPP-32 caused the reverse (Bateup *et al.*, 2010). DARP-32 is involved in a dopamine-triggered signalling cascade important for synaptic plasticity and related to drug induced neuroadaptations (Nairn *et al.*, 2004).

In optogenetic experiments a channelrhodopsin (ChR), a non-specific cation channel opened/activated by light, can be used to stimulate neurons (Boyden *et al.*, 2005) whilst halorhodopsin (NpHR), a light-activated chloride channel, is used to inhibit action potentials (Zhang *et al.*, 2007). As with other transgenes optogenetic constructs have targeted to D1 and D2 expressing neurons and localised transfection of ChR has been achieved using viral vectors. Cre-dependent ChR constructs were transfected throughout the basal ganglia of BAC mice expressing Cre in D1 or D2 neurons. Optogenetic stimulation of D1-MSNs increased locomotion and rescued the phenotype in a 6-OHDA Parkinson's model, whereas D2 activation caused freezing and bradykinesia (A. V. Kravitz *et al.*, 2010). Using the same manipulation in the ventral striatum D1 excitation was used to induce long lasting CPP while D2-MSN stimulation induced short-lived conditioned place aversion (Kravitz, Tye and Kreitzer, 2012). Additionally, operant responding was established for a lever triggering D1-MSN stimulation, whereas a lever triggering D2-MSN stimulation was avoided.

The segregation of D1 and D2 receptor expression to the direct and indirect pathways respectively is well established in the dorsal striatum however recently evidence indicates that this is not so in the ventral striatum. Kupchik and colleagues (2015) used a Cre-dependent ('floxed') channelrhodopsin (ChR2), delivered via a viral vector, to optogenetically activate D1 or D2 neurons in the NAc core of Cre-expressing transgenic mice. They recorded GABAergic IPSCs from the Ventral Pallidum (VP) following optogenetic stimulation of either population and found that that up to 50% of dorsal VP neurons are innervated by both D1 and D2 MSN afferents. We must therefore exercise caution in

referring to D1 and D2 populations as the 'direct' or 'indirect' pathways within the ventral striatum.

1.2.2.3. Electrophysiology of D1/D2 MSNs

Historically MSNs were characterised electrophysiologically by their hyperpolarized resting membrane potential and low input resistance (Kita, Kita and Kitai, 1984). The development of BAC GFP transgenic mice under the control of promoters for D1 and D2 receptors has allowed separate electrophysiological characterisation of the two populations (Gong *et al.*, 2003). Whole-cell patch-clamp recordings revealed that D2 MSNs exhibit larger excitatory postsynaptic potentials (EPSPs) and greater repetitive spiking than D1 MSNs (Kreitzer and Malenka, 2007; Bevan, Kreitzer and Berke, 2011). This may be partly explained by the greater surface area of D1- MSNs to D2-MSNs due to a greater number of primary dendrites (Gertler, Chan and Surmeier, 2008).

Early experiments using in vivo recordings of striatal MSNs also demonstrated pattern of spontaneous activity consisting of long periods of silence separated by brief episodes of firing i.e. 'irregular burst firing' (Wilson, 1993). MSNs also exhibited two preferred, subthreshold membrane potential states (C. J. Wilson and Kawaguchi, 1996). Membrane potentials alternate between a resting hyperpolarized 'down' state, between -90 and -70 mV, and a less hyperpolarized 'up' state, between -60 and -40 mV, which is only a few millivolts (3-5mV) below the spike threshold. Irregular spike discharge and spontaneous burst firing are observed only during the up state (Wilson and Groves, 1981), therefore the transition from the down state to the up state is proposed to be critical for spike firing in MSNs. Neurons may exhibit 'two-state' behaviour because of their intrinsic properties, because they are in a network that imposes it on them or both in combination. In MSNs 'two-state' behaviour arises from both their intrinsic membrane properties and phasic changes in the excitatory inputs they receive (Wilson and Kawaguchi, 1996).

MSNs express two different groups of ion channels which maintain these states dependent upon excitatory input, or lack thereof, to the MSN. During the down state, when synaptic input is low, the input resistance of MSNs is low (10-30 MOhms) creating a stable membrane potential that is relatively insensitive to

small synaptic inputs. This results from the high expression of hyperpolarization-activated KIR2 potassium channels, which move the membrane potential closer to the potassium reversal potential and therefore limit membrane depolarization (Nisenbaum and Wilson, 1995).

Shifting MSNs into the up state requires a strong, synchronous depolarizing input from cortical and thalamic afferents (Blackwell, Czubyko and Plenz, 2003). The up state is then dependent upon sustained excitatory input and is modulated by depolarization-activated potassium channels, largely in the Kv1 family (Shen *et al.*, 2003). These channels tend to return the membrane potential to the Down state or in the presence of strong excitatory synaptic input limit the sensitivity of the neuron to those inputs. This maintains membrane potential within a relatively narrow range which is marginally below the spike threshold (Shen *et al.*, 2003).

The outwardly-rectifying K⁺ channels are highly active in the Up state (Nisenbaum *et al.*, 1996), and consequently the membrane potential remains relatively constant and very large changes in synaptic input are required to achieve even small increases in depolarization. It has therefore been questioned what kind of synaptic input can trigger spiking in the up state. Computer simulations have suggested that brief depolarisations following rapidly changing in synaptic current may provide a window during which large sudden inputs can trigger a spike before voltage-sensitive channels are recruited to oppose their action (Wilson, 1995). Another possibility is that GABAergic activity may synchronise with large excitatory inputs to enable spikes to be triggered.

GABAergic activity has been revealed to be involved in the generation of the up state in striatal MSNs (Kita, 1996). Classically GABAergic inputs to MSNs have been considered inhibitory; however, activation of GABA_ARs has been demonstrated to produce excitatory effects under certain physiological conditions (Cherubini, Gaiarsa and Ben-Ari, 1991; Gullledge and Stuart, 2003). It was demonstrated that during the 'up state' MSN membrane potential is below the reversal potential of GABA_A inputs, therefore inhibition from fast-spiking interneurons results in depolarisation of MSNs (Plenz and Kitai, 1998).

1.2.3.4. Striatal Interneurons

Populations of interneurons constitute the remaining 5% of striatal neurons, these have been divided into four groups, outlined below, based on their anatomy and expression of specific histochemical markers (Kawaguchi *et al.*, 1995).

Of these, cholinergic interneurons make up 1-2% of striatal cells and are currently the most well characterised. These interneurons are relatively large (20-50µm diameter cell body) and possess widespread dendritic and axonal fields (Wilson, Chang and Kitai, 1990; Kawaguchi *et al.*, 1995). Cholinergic interneurons receive excitatory input from the thalamus and cortex, and inhibitory GABAergic inputs from MSNs (Bolam *et al.*, 1986; Chang, 1988; Brown *et al.*, 2012). Due to their varied inputs and widespread axonal fields cholinergic interneurons are able to integrate synaptic input over an extensive area. They and project to multiple MSNs each and, to a lesser extent, other striatal interneurons (Bolam, Wainer and Smith, 1984; Chang and Kita, 1992).

Importantly cholinergic interneurons have been implicated in reward-based learning and by mediating of dopamine-dependent striatal plasticity (Wang *et al.*, 2006). In vivo recordings have demonstrated that cholinergic interneurons are tonically active, exhibiting slow irregular spontaneous activity of 2-10Hz (Wilson, Chang and Kitai, 1990; Bennett, Callaway and Wilson, 2000). It has been discovered that reward related cues elicit pause in the tonic activity of cholinergic interneurons which is thought to provide a 'temporal window' during which phasic dopaminergic activity can be distinguished from tonic dopamine states (Morris *et al.*, 2004; Cragg, 2006). Thus, GABA projection neurons from the ventral tegmental area (VTA), which synapse almost exclusively on NAc cholinergic interneurons, inhibit their activity to promote stimulus-outcome learning (Brown *et al.*, 2012). Notably, cholinergic interneurons also express D2 dopamine receptors postsynaptically and therefore must be considered when interpreting experiments which pharmacologically or genetically manipulate D2Rs (Dawson, Dawson and Wamsley, 1990).

The remaining striatal interneurons are GABAergic and are further classified by their expression of (a) parvalbumin, (b) somatostatin, neuropeptide Y and nitric

oxide synthase, and (c) calretinin (Kawaguchi *et al.*, 1995). Of these parvalbumin expressing interneurons make up 1% of the striatum (Berke, 2011) and are termed fast-spiking (FS) based on their fast-firing and short duration action potentials with a short-spike after-hyperpolarization. Less is known about the function of these neurons however they receive excitatory inputs from the cortex and thalamus, and inhibitory inputs from other interneurons and a subpopulation of neurons within the globus pallidus (Chang and Kita, 1992; Bevan *et al.*, 1998; Sidibé and Smith, 1999; Ramanathan *et al.*, 2002) and in turn act in synchrony to modulate MSNs via many GABAergic synapses (Bennett and Bolam, 1994; Tepper and Koós, 1999; Kubota and Kawaguchi, 2000; Fukuda, 2009).

GABAergic interneurons expressing somatostatin, neuropeptide Y, and nitric oxide synthase exhibit low-threshold and persistent plateau depolarizations, high input resistance, and relatively depolarized resting potentials, and are termed low-threshold spiking (LTS) interneurons (Kawaguchi, 1993). LTS interneurons receive innervation from, cortex, thalamus and direct pathway MSNs and in turn project back to MSNs (Kubota *et al.*, 1988; Vuillet *et al.*, 1989). Finally, GABAergic interneurons expressing calretinin are found predominantly within the rostral-medial region of the caudate putamen, where they are proposed to act as 'calcium-buffers' stabilising the concentration of free calcium ions within MSNs (Baimbridge, Celio and Rogers, 1992; Résibois and Rogers, 1992). Calretinin expressing neurons exhibit similar electrophysiological properties to LTS interneurons, of which they can be considered a sub-type, however they receive cortical but not thalamic input (Sidibé and Smith, 1999). The physiological role of LTS neurons in the striatum has yet to be elucidated.

1.2.3.5. The Dorsal and Ventral Striatum

Classically, the striatum has been divided into two subregions, dorsal and ventral, based on the different cortical, thalamic and dopaminergic afferents to each region (McGeorge and Faull, 1989). Broadly, the dorsal striatum comprises the caudate nucleus and putamen, whereas the ventral striatum incorporates the nucleus accumbens (NAc) core and shell, olfactory tubercle, and the ventromedial portions of the caudate and putamen. It has been difficult to clearly define anatomical boundaries between these subregions therefore a

functional delineation has been proposed leading to distinction between ventromedial and dorsolateral regions within the striatum (Voorn *et al.*, 2004). This functional delineation is reflected structurally with cortical, thalamic and amygdaloid inputs into the striatum are predominantly arranged in a dorsolateral-to-ventromedial fashion.

Premotor and motor cortical areas, the mediodorsal, ventroanterior and ventrolateral thalamus, and the central nucleus of the amygdala (CeA), primarily project to the caudate nucleus and putamen. Accordingly, the dorsal striatum mediates sensorimotor control and motor planning (Kemp and Powell, 1970; Aldridge, Anderson and Murphy, 1980; McFarland and Haber, 2000). The orbital and medial prefrontal cortex, midline and medial intralaminar nuclei, and basolateral amygdala (BLA), primarily project to the ventral striatum, particularly the NAc. Accordingly, the ventral striatum mediates reward-based learning and goal-directed behaviours (Kunishio and Haber, 1994; Everitt *et al.*, 1999; Haber, 2003). The NAc also receives input from the ventral hippocampus, which mediates the development of context-based learning (Mattson *et al.*, 2007; Crombag *et al.*, 2008).

1.2.3.6. The Nucleus Accumbens (NAc)

A large body of research has demonstrated that the Nucleus Accumbens (NAc), is a critical structure for mediating the rewarding and motivational properties of drugs of abuse (Caine *et al.*, 2001; Everitt and Robbins, 2005). The NAc receives inputs from various BG nuclei, including the VTA and BLA, and from the prefrontal cortex (PFC), and in turn the NAc projects to various BG nuclei, and thalamic regions. The NAc has been proposed to be of central importance to the limbic-motor interface and thereby mediate goal-seeking actions in response to reward-predictive stimuli (Mogenson and Yang, 1991; Wise, 1998; Nicola, 2007).

All known drugs of abuse have been shown to increase dopamine release within the NAc, especially within the Shell sub-region (Wise, 1987, 1988; Di Chiara and Imperato, 1988; Pettit and Justice, 1989). Dopaminergic activity in the NAc underlies the ability of psychostimulants to increase locomotor activity. Selective lesion of dopaminergic neurons, induced by 6-OHDA, in the NAc

attenuated psychostimulant (amphetamine or cocaine) induced locomotor activity (Kelly, Seviour and Iversen, 1975; Kelly and Iversen, 1976; Joyce, Stinus and Iversen, 1983) as well as attenuating cocaine, but not heroin, self-administration (Pettit *et al.*, 1984). Dopamine transmission in the NAc also modulates reward-based learning and conditioning. Direct injection of amphetamine into the NAc, thereby increasing dopamine transmission, enhances responding for reward-related stimuli (Taylor and Robbins, 1984) and this is blocked by 6-OHDA lesion of dopaminergic neurons in the NAc (Taylor and Robbins, 1986).

The NAc can be divided into two sub-regions, the Core and Shell, which appear to have different roles in drug related behaviour. The efferent projections from the NAc differ between the Core and Shell (Heimer *et al.*, 1991; Zahm and Brog, 1992; Zahm and Heimer, 1993; Zahm, 1999). The NAc Core parallels basal ganglia circuitry by sending outputs through the ventral pallidum (dorsolateral district), subthalamic nucleus and substantia nigra, which in turn project via the motor thalamus to premotor cortical areas. In contrast, the Shell projects preferentially to subcortical limbic regions including the lateral hypothalamus, ventral pallidum and VTA (Zahm, 1999). Interestingly, there are also direct interconnections between core and shell neurons indicating that these NAc sub-regions do not function completely independently, but rather comprise interacting neuronal networks (van Dongen *et al.*, 2005).

Rats will self-administer cocaine or alcohol into the NAc Shell but not Core (Rodd-Henricks *et al.*, 2002; Engleman *et al.*, 2009). Lesions of the NAc shell were sufficient to block cocaine- and amphetamine-CPP (Sellings and Clarke, 2003; Sellings, McQuade and Clarke, 2006), whilst administration of cocaine in the NAc Shell, but not Core, facilitated psychostimulant-CPP (Liao *et al.*, 2000). Similarly, infusion of amphetamine in the NAc Shell enhanced responding for sucrose-reward-related stimuli, but did so without increasing positive hedonic reactions to the sucrose (Wyvell and Berridge, 2000).

The Nucleus Accumbens has also been found to be critical in mediating the phenomenon of behavioural sensitisation, i.e. increased behavioural effects of drugs including psychostimulants, ethanol and morphine following repeated administration (Tilson and Rech, 1973; Segal and Mandell, 1974; Cador, Taylor

and Robbins, 1991; Kalivas and Duffy, 1993; Hoshaw and Lewis, 2001). Lesions of the NAc Shell attenuated the induction of, but not expression of, behavioural sensitisation to cocaine (Todtenkopf *et al.*, 2002; Todtenkopf, Stellar and Melloni, 2002), and infusions of cocaine or amphetamine to the NAc Shell, but not Core, induced behavioural sensitisation (Pierce and Kalivas, 1997).

Further, after repeated administration of drugs of abuse (including psychostimulants, opiates and alcohol) exposure to those drugs caused greater dopamine release with the NAc (Di Chiara and Imperato, 1988; Wise, 1998). In addition, D1 dopamine receptors in the NAc show enhanced sensitivity to dopamine following repeated cocaine administration (Henry, Greene and White, 1989; Henry and White, 1991). Repeated exposure to psychostimulant drugs also produces enduring alterations in intracellular signalling pathways and structural changes in neurons within the NAc (Nestler, Barrot and Self, 2001). For example, cocaine administration increased spine density on dendrites of MSNs in the NAc Shell, but not core (Robinson and Kolb, 1999; Robinson *et al.*, 2001). The neural sensitisation of the NAc and corresponding behavioural effects are extremely robust, the enhanced behavioural response has been found to endure persistently up to a year after the final drug exposure, and possibly longer (Paulson, Camp and Robinson, 1991; Boileau *et al.*, 2006).

Robinson and Berridge (1993) have argued that repeated exposure also leads to sensitisation to the incentive motivational properties of drugs. They propose an 'incentive-sensitisation' theory of addiction whereby neural substrates mediating the attribution of incentive salience, termed 'wanting', are sensitised by repeated drug exposure. In contrast substrates which mediate the hedonic experience of a drug, termed 'liking', are dissociable and remain unsensitised or diminished (Robinson and Berridge, 2008). Given the evidence of cross-sensitisation between drugs of abuse, it has been proposed that these effects may be mediated by common neural mechanisms (Horger, Shelton and Schenk, 1990; Kalivas and Stewart, 1991; Cunningham and Kelley, 1992; McDaid *et al.*, 2005).

Supporting this theory, it has been demonstrated that sensitisation with amphetamine, cocaine, morphine or ethanol has facilitated the subsequent acquisition of self-administration or CPP produced by the same drug, or by a different drug (Lett, 1989; Horger, Shelton and Schenk, 1990; Piazza *et al.*,

1990; Mendrek, Blaha and Phillips, 1998; Hoshaw and Lewis, 2001; Camarini and Hodge, 2004; McDaid *et al.*, 2005). A dissociation between 'wanting' and 'liking' is evidenced by anatomically distinct substrates of the reinforcing effects of drugs compared with the hedonic properties of rewarding stimuli (Robinson and Berridge, 1993; Wyvell and Berridge, 2000).

GABAergic activity in the NAc has been implicated in mediating the hedonic and reinforcing properties of natural rewards as well as drugs of abuse. Injections of GABA_A receptor agonists directly into the NAc shell increased consumption of sucrose but did not affect water consumption (Basso and Kelley, 1999) whilst injection of GABA_A antagonists is sufficient to reduce alcohol consumption (George F. Koob, 2004). Injection of the GABA_A agonist muscimol into the rostral NAc shell induced CPP, whereas infusion into the caudal NAc Shell induced conditioned place aversion (Reynolds and Berridge, 2001; Reynolds, Hyland and Wickens, 2001) indicating that GABAergic receptors in those subregions of the NAc mediate important and opposing roles in associative conditioning.

1.2.4. The Ventral Tegmental Area (VTA)

The VTA is notable as the origin of dopaminergic input in the mesolimbic dopamine system. It projects to the striatum, in particular the NAc, as well as the amygdala, hippocampus and prefrontal cortex (Swanson, 1982; Albanese and Minciacchi, 1983; Ikemoto, 2007). The VTA also sends GABAergic and glutamatergic projections to the NAc and prefrontal cortex.

By various mechanisms drugs of abuse increase activation of dopamine neurons in the VTA which release dopamine to downstream targets, primarily the NAc (reviewed, Oliva and Wanat, 2016). Rodents readily self-administered cocaine, morphine, nicotine or ethanol directly into the VTA (Bozarth and Wise, 1981; Corrigall, Coen and Adamson, 1994; David *et al.*, 2004; Rodd *et al.*, 2004), and correspondingly, lesions of the VTA attenuate or abolish self-administration of cocaine and heroin (Roberts and Koob, 1982; Bozarth and Wise, 1986).

The repeated administration of cocaine, amphetamine or dopamine re-uptake inhibitors directly into the VTA initiates behavioural sensitisation (Vezina, 1996; Cornish and Kalivas, 2001). It has therefore been proposed that neuroadaptations of the VTA predominantly mediate the initiation of behavioural

sensitisation, while the NAc is required for the expression of sensitisation (White and Kalivas, no date; Kalivas and Stewart, 1991; Pierce and Kalivas, 1997).

Cocaine-evoked dopamine release was enhanced in the VTA of cocaine-sensitized animals (Parsons and Justice, 1993) and dopamine neuron excitability was enhanced in the VTA of animals with a history of repeated exposure to amphetamine, cocaine, or ethanol (White and Wang, 1984; Henry, Greene and White, 1989; Brodie, 2002). Electrophysiological studies have demonstrated that single or repeated exposure to drugs of abuse, including cocaine, amphetamine, ethanol, morphine, and nicotine, induced long-term potentiation (LTP) of VTA dopamine neurons (Ungless *et al.*, 2001; Saal *et al.*, 2003; Borgland, Malenka and Bonci, 2004).

VTA neurons have also been found to increase their firing rate in response to a conditioned stimulus previously paired with primary rewards (Schultz, Dayan and Montague, 1997; Fiorillo, Tobler and Schultz, 2003). Injection of the GABA_A agonist muscimol into the VTA abolished the ability of conditioned cues to increase instrumental responding in a test of Pavlovian to instrumental transfer (PIT), as well as decreasing cocaine-seeking maintained by conditioned reinforcers (Di Ciano and Everitt, 2004; Murschall and Hauber, 2006). *In-vivo* recordings of rats responding in an operant task revealed that injection of dopamine agonists into the VTA blocked NAc neuronal firing responses to incentive cues and behavioural responding; indicating VTA dopaminergic input to the NAc is required for goal directed behaviour (Yun *et al.*, 2004).

GABA_ARs within the VTA are likely to modulate reward processing. Ligands acting directly at GABA_ARs have been shown to be freely self-administered directly into the VTA (David *et al.*, 1997). Within the VTA GABA_ARs are predominantly located on GABAergic neurons which provide tonic inhibitory inputs onto other, dopaminergic, VTA neurons as well as projecting to the NAc and pre-frontal cortex (Johnson and North, 1992). Firing of GABA neurons in the VTA is facilitated during cues that predict appetitive rewards (Cohen *et al.*, 2012) and optogenetic experiments revealed that stimulation of VTA GABA neurons suppressed the release of DA within the NAc (van Zessen *et al.*, 2012). Thus, activity at GABA_ARs will alter neurotransmission between GABA and DA neurons

within the VTA, as well as projections fibres to the NAc, thereby modulating reward processing.

1.2.5. The Ventral Pallidum (VP)

The ventral pallidum (VP) is a central convergent point for input from the NAc, orbitofrontal and prefrontal cortex, amygdala, lateral hypothalamus, ventral tegmental area, subthalamic nucleus, and other structures related to reward (Smith *et al.*, 2009). The VP projects back to nearly all its input sources, including the NAc, for reciprocal information exchange and translates limbic motivation signals into motor output (Mogenson, Jones and Yim, 1980; Mogenson and Yang, 1991; Groenewegen, Berendse and Haber, 1993; Churchill and Kalivas, 1994).

In particular NAc to VP projections have been proposed as a major mechanism of translation of motivational signals to motor output (Mogenson and Yang, 1991). It has also been proposed that release of VP neurons from the tonic inhibition by GABAergic inputs from the NAc is a primary 'downstream' mechanism by which hyperpolarizations in the NAc stimulate reward and motivation (Smith *et al.*, 2009).

Lesions of the VP, or inactivation by the GABA_A agonist muscimol, decrease voluntary food and drink consumption, in fact replacing positive hedonic taste reactions with aversive reactions (Cromwell and Berridge, 1993; Shimura, Imaoka and Yamamoto, 2006). Conversely, GABA blockade in the VP fails to elevate hedonic reactions to taste rewards (Smith and Berridge, 2005) indicating that baseline neuronal activity in VP has a necessary role in normal hedonic valuation, although depolarization induced by GABAergic disinhibition is not sufficient to enhance hedonic valuation of food.

The VP is also involved in processing rewarding stimuli and motivated behaviour. *In-vivo* recordings have demonstrated that sucrose rewards and conditioned cues predicting sucrose both elicit phasic burst firing in the VP (Tindell, Berridge and Aldridge, 2004). Lesion or inactivation of the VP attenuated or abolished the reinforcing properties of natural rewards and drugs of abuse. This includes attenuated Pavlovian incentive learning, reducing instrumental responding for alcohol or cocaine, and blocking acquisition and expression of sucrose,

amphetamine or morphine CPP (Robledo and Koob, 1993; Harvey *et al.*, 2002; June *et al.*, 2003). Electrophysiological evidence suggests that VP neurons use separate population- and firing rate activity-patterns to distinguish enhancement of 'liking' vs 'wanting' by amphetamine and opiates (Smith *et al.*, 2009).

The VP is critically involved in the development and expression of morphine-induced behavioural sensitization and there is some evidence that it mediates psychostimulant-sensitisation. Blocking μ -opioid receptors within the VP is sufficient to block the development of sensitized motor responding to systemically administered morphine (Johnson & Napier, 2000). Dopamine release in the VP is increased by sensitisation to methamphetamine where it also induced long-lasting upregulation of pCREB and Δ FosB expression. Cross sensitisation to morphine in cocaine sensitised rats correlated with increased sensitivity of VP neurons to morphine and decreased GABAergic activity there (McDaid *et al.*, 2005). VP neurons projecting to the VTA show increased cFos expression following cue-induced reinstatement for cocaine (Mahler & Aston-Jones, 2012) and silencing these neurons via DREADDs was sufficient to blocking cue-induced reinstatement (Mahler *et al.*, 2014).

1.2.6. The Prefrontal Cortex (PFC)

The prefrontal cortex (PFC) projects to multiple sites within the mesolimbic dopamine system, including the VTA and NAc. These glutamatergic projections inducing burst firing of DA neurons in those regions (Sesack *et al.*, 1989; Chergui *et al.*, 1993; Carr and Sesack, 2000). GABAergic neurons within the PFC can also modulate NAc and VTA activity indirectly by inhibiting PFC glutamatergic afferents to various BG nuclei (Christie *et al.*, 1987; Matsumura, Sawaguchi and Kubota, 1992).

Clinically reduced volume and damage of the PFC are common features of people with addictions to illicit drugs (Liu, M.D. *et al.*, 1998) and alcohol (Pfefferbaum *et al.*, 1997). This damage is related to increased impulsivity associated with addiction (Crews and Boettiger, 2009). Neuroimaging studies reveal activation of the orbitofrontal cortex of addicted subjects during intoxication, craving, and bingeing, and deactivation during withdrawal (Goldstein and Volkow, 2002). Cognitive functions supported by neurons in the PFC are disrupted by acute and

chronic exposure to alcohol and ethanol inhibits persistent activity and spike firing of PFC neurons *in-vivo* (Tu *et al.*, 2007).

Cocaine elicits increased dopamine release in the medial PFC (mPFC) (Sorg and Kalivas, 1993). However, while the cocaine-induced increase in dopamine levels in the NAc and VTA is augmented following cocaine-sensitisation (Di Chiara and Imperato, 1988; Henry, Greene and White, 1989; Schultz, Dayan and Montague, 1997), the response of extracellular dopamine levels in the mPFC is attenuated (Prasad, Hochstatter and Sorg, 1999). Interestingly, administration of low-dose methamphetamine to the mPFC abolished behavioural sensitisation indicating decreased mPFC dopamine release contributes to behavioural sensitisation.

GABA is known to modulate dopamine and glutamatergic systems in the mPFC. In cocaine-sensitised rats a challenge injection of cocaine, but not saline, resulted in a significant increase in extracellular GABA levels in the mPFC at 1 and 7 days but not 28 days following repeated cocaine exposure (Jayaram and Steketee, 2005). Morphine-induced CPP was potentiated or attenuated by GABA_AR agonists and antagonists respectively (Rozeske *et al.*, 2009).

1.2.7 The Amygdala

Classically the amygdala has been associated with the modulation of memory consolidation and emotional learning, including appetitive and fear conditioning (Everitt, Cardinal, Parkinson, & Robbins, 2003; Gallagher, Graham, & Holland, 1990; McGaugh, 2002; McIntyre *et al.*, 2003; Wilensky, Schafe, & LeDoux, 2000).

The Central Amygdala (CeA) has been highly implicated as a critical locus of neuroadaptation during transition to alcohol dependence (Roberto, Gilpin, & Siggins, 2012). The CeA is composed mostly of GABAergic projection neurons and interneurons (Sun & Cassell, 1993; Veinante & Freund-Mercier, 1998). Acute alcohol increases GABAergic synaptic transmission in the CeA (Roberto *et al.*, and BLA (Zhu and Lovinger, 2006) via increased presynaptic GABA release. Further, *in-vitro* electrophysiological results show that chronic alcohol exposure augments baseline GABA release in the CeA (Roberto *et al.*, 2004). Infusion of a GABA_AR antagonists directly into the amygdala suppresses drinking by alcohol-dependent rats without affecting intake by nondependent controls (Roberts, Cole

and Koob, 1996). Injection of GABA_AR antagonists more specifically in the CeA suppressed alcohol drinking by nondependent rats as well (Hyytiä and Koob, 1995).

The BLA projects heavily to the NAc as well as the medial PFC (mPFC) and hippocampus, and is proposed to play an important role in mediating motivational behaviour (Balleine, Killcross, & Dickinson, 2003; Everitt et al., 2003). The BLA forms a cortico-subcortico 'loop' with the mPFC which is proposed to be important for integrating affective information with stimulus properties, thus mediating stimulus-outcome associations (Everitt et al., 2003; Quirk et al., 2003). The BLA also sends glutamatergic projections to NAc MSNs which are proposed to modulate incentive motivational properties of reward-associated stimuli (Quirk et al., 2003b; Stuber et al., 2011).

Lesions of the BLA attenuate sucrose, cocaine or morphine CPP, as well as attenuating cue-induced reinstatement of cocaine self-administration (Everitt, Morris et al., 2002; Milekic et al., 2006; Nicola, 2004). *In-vivo* recordings during operant tasks have been used to demonstrate that BLA neurons fire phasically in response to reward-predictive cues (Tye & Janak, 2007; Uwano et al., 1995). Increased extracellular dopamine was observed in the BLA of rats during a discriminative operant task (Hori, Tanaka and Nomura, 1993; Weiss *et al.*, 2000) and injection of dopamine agonists into the BLA has also been shown to enhance appetitive Pavlovian conditioning in a discriminative approach task (Hitchcott, Bonardi and Phillips, 1997).

Lesion of the BLA did not affect the development of normal conditioned responding to a stimulus paired with a food reward (Hatfield et al., 1996) however it abolished the ability of rats to adjust responding to the conditioned stimuli when the reward was devalued (Balleine, Killcross and Dickinson, 2003). This suggests that the BLA is necessary for encoding the value of a rewards associated with conditioned stimuli.

Interestingly, D1- and D2-expressing neuronal populations in the BLA appear to mediate drug reinforcement via mechanisms which are dependent upon prior drug experience. Intra-BLA injection of D1 but not D2 receptor antagonists blocked morphine CPP in drug-naïve rats whereas the reverse, D2 but not D1

receptor antagonists, blocked CPP in drug-dependent animals in drug withdrawal (Lintas *et al.*, 2011). In this study the affective doses also reduced neuronal firing in the NAc shell suggesting this is an important pathway in opiate reinforcement.

Lesions of the amygdala have had mixed effects on behavioural sensitisation to amphetamine, either blocking or having no effect on the development of sensitization (Cador *et al.* 1999; Wolf *et al.*, 1995). The BLA may mediate adaptive changes in response to drug related stimuli. Cocaine-seeking behaviour elicited by cocaine-paired environmental stimuli was found to be associated with an increase in cFos expression in the BLA, as well as the NAc and hippocampus (Neisewander *et al.*, 2000).

1.2.8. The Hippocampus

The hippocampus underlies learning of associations between environmental contexts and rewarding or aversive stimuli. Environmental 'triggers' are known to induce drug seeking behaviour and relapse (Robbins and Everitt, 2002). Lesion of the hippocampus shortly prior to foot-shock conditioning prevented the expression of conditioned fear in an associated environment (Kim and Fanselow, 1992). This effect was observed when lesions were made 1 day, but not at 28 days, after training which indicates formation but not expression of the association is affected.

Similarly, lesions or pharmacological inactivation of the hippocampus abolish both the acquisition and expression of cocaine or morphine CPP (Meyers, Zavala, & Neisewander, 2003; Meyers *et al.*, 2006; Milekic *et al.*, 2006) and impair acquisition of operant cocaine self-administration (Caine *et al.*, 2001). Cocaine-seeking behaviour elicited by cocaine-paired environmental stimuli was found to be associated with an increase in cFos expression in the hippocampus (Neisewander *et al.*, 2000).

Stimulation of the ventral subiculum of the hippocampus induced dopamine (DA) release within the NAc via increased firing of dopaminergic VTA projections (Brudzynski and Gibson, 1997; Legault, Rompré and Wise, 2000). Stimulation of the hippocampus also potentiated the ability of contextual cues to reinstate drug seeking behaviour following extinction of operant cocaine-self administration in

rats. Accordingly, this effect was blocked by pharmacological inactivation of the hippocampus (Luo, Callaway, & Svoboda, 2008; Vorel et al., 2001). Notably this effect was also dependant on glutamatergic input from the VTA.

Lesion of the hippocampus abolished the potentiating effect of intra-NAc amphetamine on locomotor activity (Burns, Robbins and Everitt, 1993) and inactivation of the dorsal, but not the ventral, hippocampus blocked the expression of amphetamine sensitization (Degoulet et al., 2008). The dorsal and ventral hippocampus have been implicated in context- and cue-dependent reinstatement, respectively (Fuchs et al., 2005; Sun & Rebec, 2003) indicating this effect may be due to the modulating effect of environmental contexts in sensitisation.

Behavioural sensitization to amphetamine has also been associated with enhanced ventral hippocampal modulation of dopamine neuronal activity (Lodge and Grace, 2008; Britt *et al.*, 2012) demonstrated that long-term withdrawal from repeated cocaine administration produced an increase in synaptic strength selectively in the ventral hippocampal input to the NAc shell. Furthermore, inactivation of the said input to the NAc during attenuated cocaine-induced locomotion in a specific environment.

1.3. GABA_A Receptors

1.3.1. The Structure of GABA_A receptors

GABA receptors fall into two classes: GABA_A and GABA_B. GABA_A receptors (GABA_ARs) are ligand gated ion channels i.e. ionotropic receptors, whereas GABA_B receptors (GABA_BRs) are G protein-coupled receptors i.e. metabotropic receptors. Here we will focus primarily on GABA_ARs.

GABA_ARs are heteropentameric chloride channels, consisting of five heterogenous protein subunits arranged around a central pore. They belong to a large 'super-family' of evolutionarily and structurally related cys-loop ligand-gated ion channels which includes nicotinic acetylcholine receptors, glycine receptors, and the 5-HT₃ receptor (Goetz et al., 2007). These can be further classified into several isoforms based on the different combination of subunits present in each, i.e. their stoichiometry.

In humans there currently known to be 18 GABA_AR subunits, which can be divided by sequence homology into seven subunit categories. There are six α subunits ($\alpha 1-6$), three β subunits ($\beta 1-3$), three γ subunits ($\gamma 1-3$), three ρ subunits ($\rho 1-3$), and one each of the ϵ , δ , θ , and π subunits. Five subunits can combine in different ways to form GABA_A receptors with the minimum requirement both α and β subunits (Baumann, Baur, & Sigel, 2001). Despite the potential for vast numbers of individual receptor isoforms GABA_A receptors typically consist of two α -subunits, two β -subunits and either a γ - or δ -subunit (Whiting, McKernan and Wafford, 1995; Sieghart, 2006).

GABA_AR subunits consist of four hydrophobic transmembrane domains (TM1–4) made up of ~ 20 amino acids with both the N- and C-terminus located extracellularly. Of these, the TM2 domain lines the pore of the channel (Jacob, Moss and Jurd, 2008). GABA binds to a site at the large extracellular N-terminus at the interface between an α and β subunit where the binding of two GABA molecules induces channel opening (Baumann, Baur, & Sigel, 2003). The binding sites for several psychoactive drugs also fall within the extracellular N-terminus such as benzodiazepines (between α and γ) and barbiturates (between α and β) (Johnston, 2005).

Selective assembly of GABA_AR isoforms occurs within the endoplasmic reticulum and can therefore be selectively expressed and targeted to specific subcellular localities. Differences in subunit composition, i.e. subtype, confer differences in receptor, location, function, physiology and pharmacological properties (Goetz *et al.*, 2007).

1.3.2. Synaptic and Extrasynaptic GABA_A receptors

Historically it was observed that GABA_ARs mediate inhibition via fast 'phasic' transmission of activity occurring within the synapse. Subsequently, certain GABA_AR isoforms have been physiologically characterised which are commonly located extrasynaptically, either perisynaptically or distant from synapses. Typically, synaptic GABA_ARs comprise of $\alpha 1$, $\alpha 2$, or $\alpha 3$ in combination with $\beta 2/3$ and $\gamma 2$ subunits, whereas tonic extrasynaptic GABA_ARs predominantly comprise of $\alpha 4$, $\alpha 5$ or $\alpha 6$, coupled with $\beta 2/3$ and δ subunits, although there is some

evidence of $\alpha 1$ and γ within extrasynaptic GABA_ARs (Barnard et al., 1998; Mortensen et al., 2010).

Because GABA_AR receptors are expressed in many different subcellular and anatomical locations the concentration of GABA each population is exposed to will also vary widely. Accordingly, the GABA_AR sub-types have different pharmacological properties including sensitivity to both endogenous molecules, such as GABA and neurosteroids, and exogenous drugs (Baumann et al., 2001; Johnston, 2005; Mortensen et al., 2010).

Since they are not located at the synapse, where high concentrations of GABA are available intermittently during phasic transmission, extrasynaptic GABA_ARs are adapted to be sensitive to low levels of ambient or 'spill-over' GABA which generates a sustained 'tonic' form of inhibition (Brickley & Mody, 2012; Farrant & Nusser, 2005; Wei et al., 2003).

The α subunit has been most highly implicated in GABA sensitivity of synaptic receptors. By combining radioligand binding and electrophysiology with mutagenesis it has been possible to identify four amino acids in the extracellular N-terminal region of α subunits which largely determine GABA sensitivity of typical $\alpha\beta 3\gamma 2$ GABA_ARs (Böhme, Rabe and Lüddens, 2004). Studies manipulating the α subunit reveal EC₅₀ values (concentration of a drug that elicits half-maximal response) of the GABA-induced chloride current to vary between <1 to >50 μ M, with a rank order $\alpha 6 > \alpha 1 > \alpha 2 > \alpha 4 > \alpha 5 > \alpha 3$ (Böhme, Rabe and Lüddens, 2004; Minier and Sigel, 2004). Despite this ranking, sensitivity to GABA is increased in extrasynaptically located $\alpha 4\beta\delta$ extrasynaptic GABA_ARs compared to synaptic $\alpha 4\beta\gamma 2$ GABA_ARs indicating that other subunits contribute to increased sensitivity of extrasynaptic receptors (Mortensen et al., 2010; Jensen et al., 2013).

Extrasynaptic receptors have a higher affinity to GABA and slower desensitisation in comparison to their synaptic counterpart (Belelli et al., 2009). Furthermore, $\alpha 4\beta 3\delta$ extrasynaptic GABA_ARs are also differentially sensitive to a number of allosteric modulators and neurosteroids (Lambert et al., 2003).

Compared with synaptic GABA_ARs, δ subunit-containing extrasynaptic receptors are highly sensitive to low, physiologically relevant concentrations of

neurosteroids. This has been demonstrated by experiments on recombinant receptors (Belelli *et al.*, 2002; Wohlfarth, Bianchi, & Macdonald, 2002) and the reduced behavioural sensitivity of δ subunit knock-out mice to endogenous and synthetic neuroactive steroids (Mihalek *et al.*, 1999).

Similarly, the GABA_A agonist muscimol has a higher potency at extrasynaptic $\alpha 4\beta 3\delta$ GABA_ARs compared to synaptic $\alpha 4\beta 3\gamma 2$ and $\alpha 1\beta 3\gamma 2$ GABA_ARs, though this difference may be caused by reduced desensitisation (Mortensen *et al.*, 2010).

Extrasynaptic GABA_ARs are typically insensitive to benzodiazepine agonists (Belelli *et al.*, 2009) but can be activated by low concentrations of drugs acting as super-agonists at the GABA-binding sites, as well as taurine and γ -hydroxybutyrate (Jia *et al.*, 2008; Halonen *et al.*, 2009; Herd *et al.*, 2009; Wafford *et al.*, 2009; Absalom *et al.*, 2012).

Notably, Gaboxadol® (THIP; 4,5,6,7-tetrahydroisoxazolo-5,4-c]pyridin-3-ol), acts as a high-efficacy 'super-agonist' at δ -containing extrasynaptic GABA_ARs where it increases the frequency and duration of channel opening. In contrast, on $\alpha\beta\gamma$ -type synaptic receptors THIP has only partial agonist activity (Ebert *et al.*, 1994; Mortensen *et al.*, 2004).

More recently a novel, highly specific, positive allosteric modulator of δ -containing GABA_ARs has been created: delta-selective compound 2 (DS2). An *in-vitro* concentration-response curve indicates that DS2 produces a similar peak stimulated inhibitory current as THIP in $\alpha 4\beta \delta$ receptors; however, unlike THIP DS2 does not produce any response in $\alpha 4\beta \gamma 2$ or $\alpha 1\beta \gamma 2$ receptors even at high doses (Jensen *et al.*, 2013; Mortensen *et al.*, 2010).

1.3.3. Differential targeting of Synaptic and Extrasynaptic GABA_A receptors

Subunit composition also appears to direct the sub cellular localisation of GABA_ARs. More than 20 intracellular or transmembrane proteins have been identified which interact with TM3–TM4 intracellular loops, often very specifically with a certain subunit, to regulate the surface expression of receptors (Uusi-Oukari and Korpi, 2010).

Targeted deletion of $\gamma 2$ results in reduced synaptic GABA_AR clustering indicating its role in localising synaptic receptors. The $\gamma 2$ subunit mediates this via complex interactions with synaptic scaffolding proteins gephyrin, collybistin and neuroligin-2 (Essrich et al., 1998; Pouloupoulos et al., 2009). However, it appears that combination with other subunits in certain isoforms may nullify the synaptic anchoring properties of the $\gamma 2$ subunit which has also been found to couple with $\alpha 5$ and $\alpha 6$ within extrasynaptic GABA_ARs in hippocampal and cerebellar granule cells, respectively (Crestani et al., 2002; Wisden et al., 2002). The δ subunit is expressed exclusively in extrasynaptic GABA_ARs (Belelli et al., 2009) however there are extrasynaptic receptors lacking δ subunits, such as $\alpha 5\beta\gamma 2$ receptors. Together these receptors demonstrate that $\gamma 2$ and δ subunits cannot be solely responsible for guiding GABA_A-R targeting (Brünig et al., 2002; Glykys et al., 2007; Serwanski et al., 2006).

More recent studies found that different α subunits also direct GABA_AR localisation. The $\alpha 2$ subunit has been demonstrated to bind directly to gephyrin (Tretter et al., 2008). When molecularly engineered to pair with the same chimeric δ - $\gamma 2$ subunit, different α subunits ($\alpha 2$ versus $\alpha 6$) dictated synaptic versus extrasynaptic targeting respectively (Wu et al., 2012). Additionally, when they were engineered to interact with gephyrin $\alpha 6\beta\delta$ receptors were recruited to synaptic sites. Thus, synaptic GABA_AR targeting is controlled by specific subunit composition and the ability to interact with gephyrin.

As well as targeting GABA_ARs to the synapse accessory proteins are also able to modify channel kinetics. GABA_A receptor-associated protein (GABARAP) binds and links the intracellular domains of $\gamma 2$ subunits which promotes clustering of GABA_ARs and thereby induces cooperative opening and closing of channels (Chen et al., 2000).

Comparatively, less is known about the targeting of extrasynaptic receptors. The extrasynaptic localization of receptors containing the $\alpha 4$, $\alpha 5$, and δ subunits appears to depend on structural motifs that either prevent interaction with the postsynaptic scaffolding molecule gephyrin or allow interaction with radixin - an actin-binding protein. Extrasynaptic receptors ($\alpha 4\beta\delta$) do not colocalise with gephyrin (Crestani et al., 2002; Goetz et al., 2007; Kralic et al., 2006). $\alpha 5$ -

GABA_ARs were found to interact with radixin which thereby anchors them at extrasynaptic sites (Loebrich et al., 2006).

1.3.4. Expression of GABA_AR subunits in the mammalian brain

Immunocytochemical and *in-situ*-hybridisation analysis has revealed that GABA_A receptor subunit isoforms each exhibit unique distributions throughout the brain (Laurie, Seeburg, & Wisden, 1992; Laurie, Wisden, & Seeburg, 1992; Wisden et al., 1992).

α1 and α2 subunits are found extensively throughout the brain, with α1 being the most abundantly expressed (Pirker et al., 2000; Wisden et al., 1991). The α3 subunit isoform is localised to the cerebral cortex, olfactory bulb and brain stem nuclei (Persohn, Malherbe, & Richards, 1992; Pirker et al., 2000; Wisden et al., 1991). The α4 subunit is most highly expressed in the thalamus and otherwise distributed throughout the NAc, hippocampus, neocortex and caudate-putamen. Within the striatum it is most highly expressed in the NAc (Pirker et al., 2000; Schwarzer et al., 2001; Wisden et al., 1991). The α5 subunits is highly expressed within the hippocampus, moderately expressed within the hypothalamus, neocortex and olfactory bulb, and slightly expressed within the striatum (Ade et al., 2008; Laurie et al., 1992; Mendez et al., 2013; Persohn et al., 1992). Finally, the α6 subunit is expressed specifically in the cerebellar granule cells, hippocampal pyramidal neurons, and cochlear nucleus granule cells (Wisden et al., 2002; Wisden et al., 1991).

All three β subunits are found throughout the brain and their distribution often overlaps (Pirker et al., 2000). β1 subunits are most highly expressed in the hippocampus and olfactory bulb, and to a lesser extent in the cerebral cortex, cerebellum, superior colliculus and substantia nigra (Persohn, Malherbe and Richards, 1992; Wisden et al., 1992). β2 subunits are widely expressed, most highly in the pallidum and thalamus, and their distribution often overlaps with the α1 subunit (Moreno, et al., 1994; Pirker et al., 2000; Wisden et al., 1992). Finally, the β3 subunit is most highly expressed in the striatum and its distribution often overlaps with the α2 subunit (Miralles, et al., 1999; Pirker et al., 2000).

$\gamma 1$ subunit expression is minimally expressed throughout the brain and most highly expressed in the bed nucleus of the stria terminalis (Pirker *et al.*, 2000). In contrast, $\gamma 2$ subunits are highly expressed almost ubiquitously in the brain (Wisden *et al.*, 1992; Pirker *et al.*, 2000). The $\gamma 3$ subunit is also expressed at low levels throughout the brain (Pirker *et al.*, 2000).

The δ subunit is most highly expressed in the cerebellar granule cells, and moderately expressed in the thalamus, striatum, hippocampal dentate granule cells and neocortex (Persohn *et al.*, 1992; Schwarzer *et al.*, 2001; Wisden *et al.*, 1992). GABA_AR δ subunits, which are expressed exclusively in extrasynaptic GABA_ARs, partner either with $\alpha 6$ or $\alpha 4$ subunits in the cerebellum and forebrain respectively (Jones *et al.*, 1997; Peng *et al.*, 2002).

Expression of ρ subunits is restricted to cerebellum, colliculi and retina (Boue-Grabot *et al.*, 2002; Alakuijala *et al.*, 2005). The θ and ϵ subunits show remarkably overlapping expression throughout the brain and typically form receptors with $\alpha 3$ subunits. They are most highly expressed in the dorsal raphe and the locus coeruleus (Bonnert *et al.*, 1999; Pape *et al.*, 2009). As yet, there is no evidence of η subunit expression within the mammalian CNS, but it is known to be highly expressed within the uterus (Hedblom and Kirkness, 1997).

1.4. Ethanol

1.4.1. Neurobiology of ethanol abuse

The neurobiology of alcohol abuse has been difficult to dissect as alcohol mediates its effects via a wide range of molecular targets (Ron and Barak, 2016). Studies suggest that dopamine also has a role in the incentive motivation associated with acute alcohol intoxication. For example, direct injections of dopamine antagonists into the NAc attenuate alcohol consumption (Hodge, Samson, & Chappelle, 1997; Rassnick, Pulvirenti, & Koob, 1992). Furthermore, voluntary alcohol consumption and alcohol predictive cues induce dopamine release in the NAc (Weiss, *et al.*, 1993). However, lesions of the mesolimbic dopamine system do not completely abolish alcohol-reinforced behaviour, indicating that dopamine is an important, but not essential, component of alcohol reinforcement (Rassnick, Stinus and Koob, 1993).

Alcohol reinforcement is mediated in part by the release of endogenous opioids in the brain. Opioid antagonists acting either at all opioid receptor subtypes or only at specific subtypes suppress alcohol drinking in a variety of species and models (reviewed, Ulm et al. 1995). Furthermore, genetic deletion of the μ -opioid receptor blocks alcohol self-administration in transgenic mice (Roberts et al., 2000). Naltrexone, a subtype-nonspecific opioid receptor antagonist, is currently used as a treatment for alcoholism. Opioid receptor antagonists interfere with alcohol's rewarding effects by acting on sites in the VTA, NAc, and central nucleus of the amygdala (Koob, 2003, 2014).

Research on two of the major neurotransmitter systems in brain, GABA and glutamate, has identified key pathways for ethanol-induced intoxication and reinforcement. Ethanol has been found to inhibit the function of NMDA receptors, in a selective manner, to reduce calcium entry into neurons. Electrophysiological assessment of the effects of ethanol on NMDA receptor-mediated demonstrated that in the hippocampus and striatum concentrations of ethanol as low as 10–25mM were able to significantly inhibit NMDA receptor function (Hoffman et al., 1989; Lovinger, White, & Weight, 1989; Woodward & Gonzales, 1990). The effect of ethanol on NMDA receptor function is only modestly influenced by subunit composition (Honse et al., 2004). Behaviourally injection of glutamate antagonists into the NAc have been found to reduce ethanol self-administration (Rassnick, Pulvirenti and Koob, 1992).

1.4.2. GABA_A receptors and Ethanol

Historically, GABAergic systems were predicted as a mediator of ethanol's action due to similarities between the behavioural effects of ethanol and benzodiazepines (reduced anxiety, sedation, anticonvulsant actions, produce tolerance/addiction), which are known to act through sites on GABA_A receptors (Johnston, 2005).

There is some convergence of pre-clinical evidence that agonism of GABA_B receptors reduces alcohol consumption, motivation and withdrawal. Intraperitoneal (IP) injection of the GABA_B receptor agonist baclofen dose dependently reduced ethanol consumption in rats (Colombo *et al.*, 2000). IP baclofen and SKF 97541 (another GABA_B agonist) also reduced operant self-

administration of ethanol but also saccharin solution in mice (Besheer, Lepoutre and Hodge, 2004).

In contrast opposite effects have been found using modulators of GABA_A receptor activity. The GABA_A antagonist RO15-4513 reduced ethanol self-administration in alcohol preferring rats (Rassnick *et al.*, 1993). Ethanol consumption in rats was decreased by IP injection of the GABA_A antagonist picrotoxin but was increased by the GABA_A agonist THIP (Boyle *et al.*, 1993) indicating bidirectional control of ethanol consumption via GABA_ARs.

The effects of GABAergic compounds on ethanol consumption can be localised to specific brain regions. Alcohol drinking has been suppressed by GABA_A and GABA_B receptor antagonists when injected in the NAc, VP, bed nucleus of the stria terminalis (BNST), and amygdala (reviewed, Koob, 2004). Injections of the potent GABA_A antagonist SR 95531 into the central amygdala (CA) or Nucleus accumbens (NAc) were sufficient to suppress responding in ethanol self-administration (Hyytiä and Koob, 1995). Similarly, infusion of bicuculline or muscimol into the NAc suppressed ethanol self-administration.

1.4.3. Are GABA_A receptors sensitive to ecologically valid doses of ethanol?

Although GABA_ARs have been suggested to represent a primary target for ethanol, the direct effects of ethanol at postsynaptic receptors are achieved only at high concentrations (>40mM) unlikely to be achieved by social drinkers (Weiner and Valenzuela, 2006) which approaches the median lethal BEC in humans of ~72mM (Koski, Ojanperä and Vuori, 2002). Extrasynaptic GABA receptors were proposed as a target for ecologically valid doses of ethanol when it was observed that δ subunit-containing GABA_ARs are sensitive to much lower concentrations of ethanol (3 to 30mM) than GABA_ARs containing the γ subunit (Wallner, Hanchar and Olsen, 2003). As the γ subunit is necessary for the actions of benzodiazepines (Johnston, 2005) this presented a surprising differentiation of the actions of ethanol and benzodiazepines on GABA_ARs

However, electrophysiological evidence for the effects of ethanol on $\alpha 4\beta\delta$ receptors is mixed. Early results found that low, ecologically valid doses of

ethanol enhanced GABA_A gated current in *Xenopus laevis* oocytes expressing $\alpha 4\beta\delta$ receptors and that $\alpha 4\beta 3\delta$ were uniquely sensitive to doses as low as 1mM (Sundstrom-Poromaa *et al.*, 2002; Wallner, Hanchar and Olsen, 2003). This technique was also used to demonstrate that RO15-4513 blocked ethanol enhancement of $\alpha 4\beta\delta$ mediated current at doses that did not reduce GABA gated chloride current (Wallner, Hanchar and Olsen, 2006) suggesting a mechanism for the ability of RO15-4513 to reduce ethanol self-administration (Rassnick *et al.*, 1993). These results are controversial as they are variable and have failed to replicate. Even in experiments which supported this model the ethanol concentration required for similar enhancement of current varied from 1mM (Sundstrom-Poromaa *et al.*, 2002) to 30mM (Wallner, Hanchar and Olsen, 2003). Independent experiments on *Xenopus* oocytes and human fibroblasts expressing rat, murine or human GABA_A $\alpha 4/b3/\delta$ subunits reported that high doses of ethanol were required to enhance current; 100mM in oocytes and 300mM in fibroblasts (Borghese *et al.*, 2006). The Blood Ethanol Content (BEC) used to define 'binge drinking' equates to approximately 17mM (NIAAA, 2004) so these results are unlikely to be physiologically relevant.

The function of GABA_A receptors also is regulated by molecules known as neuroactive steroids (Lambert *et al.*, 2003) that are produced both in the brain and in other, peripheral organs. Alcohol increases the brain levels of many neuroactive steroids (Morrow *et al.*, 2001). Interestingly, this increased activity of neuroactive steroids in the brain following alcohol exposure is not dependent on their production by peripheral organs (Sanna *et al.*, 2004). IP ethanol administration was found to greatly increase levels of the neurosteroid allopregnanolone (ALLOP) in the brain and this increase was much larger in alcohol-preferring relative to non-preferring rats (Barbaccia *et al.*, 1999). IP injection of ALLOP has also been found to enhance ethanol consumption during operant self-administration and 'two-bottle choice' procedures in rats (Janak, Redfern, & Samson, 1998) and C57BL/6J mice (Sinnott, Phillips and Finn, 2002) respectively. Together, these findings suggest that neuroactive steroids are potential key modulators of altered GABA function during the development of alcohol dependence, likely acting directly at GABA_A receptors.

The ability of alcohol to facilitate GABA neurotransmission may be limited by GABA feedback onto presynaptic GABA_BRs (Ariwodola & Weiner, 2004; Wan et al., 1996). For example, acute alcohol facilitates GABAergic transmission in hippocampus (Wu & Saggau, 1994) and NAc (Nie, Madamba, & Siggins, 2000) only if GABA_B receptors are blocked.

1.4.4. Different GABA_AR isoforms affect alcohol consumption

Specific, although low-efficacy, agonists of GABA_ARs containing the α 1-subunit suppress alcohol drinking and seeking when they are injected into the CeA and VP (Harvey et al. 2002; June et al. 2003). In contrast, deletion of the α 5 subunit had no effect on alcohol consumption or reinforcement (Stephens et al., 2005).

Human genetic studies revealed that single nucleotide polymorphisms (SNPs) in the GABRA2 gene encoding the GABA_A receptor α 2 subunit are associated with alcohol dependence (Edenberg *et al.*, 2004). In humans GABRA2 polymorphisms have been linked to the subjective response to ethanol, including the hedonic value of alcohol (Pierucci-Lagha *et al.*, 2005; Haughey *et al.*, 2008). In pre-clinical studies deletion of the *gabra2* gene in mice resulted in hypersensitivity to the acute effects of ethanol (sedation and ataxia) but did not alter ethanol self-administration suggesting α 2-GABA_ARs are not critically involved not in ethanol reinforcement but in its acute effects (Dixon *et al.*, 2012).

The selective α 4 β δ super-agonist THIP has been found to modulate ethanol consumption but with mixed results when injected IP, either reducing or increasing consumption (Boyle et al., 1993; Ramaker, Strong, Ford, & Finn, 2012). When infused directly into the NAc THIP was found to attenuate drinking in mice; however, we have observed contradictory results under similar experimental conditions (Ramaker *et al.*, 2015, MacPherson, Stephens and King unpublished data). Deletion of the GABA_A δ subunit in mice reduced ethanol consumption (Mihalek *et al.*, 2001) and several studies have found that α 4 β δ receptors in the NAc shell mediate alcohol consumption and reinforcement. Virally mediated knockdown of δ or α 4 subunits in the NAc shell, but not core, reduced ethanol consumption and operant self-administration by rats (Nie, et al. 2011; Rewal et al., 2009, 2012). Together these phenotypes strongly imply that α 4 β δ receptors in the NAc have a major role in the reinforcing effects of alcohol.

1.4.5. Ethanol regulation of GABA_A gene expression

Chronic alcohol exposure also leads to alterations in the GABA_A systems. In some brain regions, alcohol affects the expression of genes that encode components of the GABA_A receptors. This has been demonstrated by changes in the subunit composition of the receptor in those regions, the most consistent of which are decreases in $\alpha 1$ - and increases in $\alpha 4$ -subunits (Devaud et al., 1997; Mhatre et al., 1993). Analysis of post-mortem PFC tissue indicated increased GABRA2 mRNA levels in alcohol-dependent individuals (Haughey *et al.*, 2008). Extrasynaptic $\alpha 4\beta\delta$ receptors were found to be rapidly upregulated GABA receptors following high-dose or chronic ethanol administration in rats (Liang *et al.*, 2007).

1.4.6. Investigating the role of $\alpha 4$ -GABA_ARs in binge drinking

We wished to examine whether $\alpha 4$ -GABA_ARs are also important in 'binge-drinking' - a pattern in which more alcohol is consumed raising blood alcohol levels to ≥ 0.08 grams of alcohol per decilitre blood (0.8mg/ml). Binge-drinking is associated with alcoholism as well as a large proportion of alcohol related deaths, diseases and social harms (NIAAA, 2004).

Previous experiments examining the role of $\alpha 4$ -GABA_ARs in ethanol consumption (described above) have primarily focused on 'two-bottle choice' or operant self-administration during which mice generally drink moderate amounts of alcohol (reviewed, Crabbe et al. 2011). Drinking in the Dark (DID) is a limited access procedure in which the water supply of a rat or mouse is exchanged for an ethanol solution (10 to 20% ABV) during a two-hour period in the dark phase of the light cycle and consumption is recorded (Rhodes et al., 2005; Ryabinin et al., 2003). Ethanol consumption in these studies was high enough to reliably predicted BECs $> 1\text{mg/ml}$ in C57BL/6J mice. DID procedures have been used to distinguish differences in alcohol drinking phenotypes between different strains and genotypes of mice (Rhodes *et al.*, 2007).

The effects of these receptors may also depend on expression within different neuronal classes and subtypes. The D1 and D2 MSN pathways appear to have different and opposing roles mediating the rewarding properties of drugs (reviewed in Chapter 1/2 and Lobo and Nestler, 2011). Multiple experiments

have found that systemically D1 and D2 agonists reduce ethanol preference and consumption (Cohen, Perrault, & Sanger, 1999; Linseman, 1990; Silvestre, O'Neill, Fernandez, & Palacios, 1996). We therefore also wish to examine whether $\alpha 4$ -GABA_ARs on D1 or D2 expressing neurons specifically have distinct roles in alcohol consumption.

1.5. Psychostimulants

1.5.1 Neurobiology of Psychostimulants

Psychostimulants, such as amphetamine and cocaine, are psychoactive drugs which induce a variety of physiological effects within the central and peripheral nervous systems, ultimately increasing psychomotor activity. Psychostimulants, mediate their effects by increasing dopamine transmission within the NAc through a variety of mechanisms (Di Chiara and Imperato, 1988; Wise and Rompre, 1989). Amphetamines inhibit monoamine oxidase (MAO) thereby preventing the breakdown of excess dopamine (Mantle, Tipton and Garrett, 1976). In addition amphetamine entering neurons indirectly causes phosphorylation of the dopamine transporter (DAT) resulting in reversal of dopamine transport (Miller, 2011). Cocaine binds to and inhibits the dopamine Transporter (DAT) (Beuming *et al.*, 2008). This results in an increased accumulation of dopamine within the synaptic cleft prolonging stimulation of postsynaptic dopamine receptors that is thought to alter the influence of excitatory neuronal inputs.

1.5.2. Behavioural Sensitisation to Psychostimulants

Behavioural sensitisation is a phenomenon whereby the stimulant effects of a drug are enhanced following repeated, intermittent administration (Robinson & Becker, 1986; Segal & Mandell, 1974; Tilson & Rech, 1973). Psychostimulants are particularly robust in their ability to induce behavioural sensitisation and the neuroadaptations which underlie it. Behavioural sensitisation to amphetamine was demonstrated to persist undiminished for over a year (Robinson & Berridge, 1993).

Repeated exposure to psychostimulant drugs produces enduring alterations in intracellular signalling pathways and structural changes in neurons within the NAc (Nestler, Barrot, & Self, 2001). For example, cocaine administration

increased spine density on dendrites of MSNs in the NAc Shell, but not core (Robinson & Kolb, 1999; Robinson et al., 2001). Repeated intermittent cocaine administration is associated with an elevated basal extracellular level of dopamine within the NAc (Di Chiara and Imperato, 1988; Kalivas and Duffy, 1990). In addition, D1 dopamine receptors in the NAc show enhanced sensitivity to dopamine following repeated cocaine administration (Henry, Greene and White, 1989; Henry and White, 1991).

As outlined earlier, the NAc and VTA are critical in mediating the induction and expression of behavioural sensitisation respectively (Kalivas & Stewart, 1991; Pierce & Kalivas, 1997; White & Kalivas, 1998). Repeated intra-VTA, but not intra-NAc, injections of psychostimulants potentiate the locomotor stimulant effects of a systemic or intra-NAc challenge of the same or other such psychostimulants demonstrating the expression of sensitisation (Cornish & Kalivas, 2001; Dougherty & Ellinwood, 1981; Hooks et al., 1992; Kalivas & Weber, 1988). Lesions of the NAc Shell attenuated of the induction of, but not expression of, behavioural sensitisation to cocaine (Todtenkopf et al., 2002; Todtenkopf, Stellar, & Melloni, 2002), and infusions of cocaine or amphetamine to the NAc Shell, but not Core, induced behavioural sensitisation (Pierce, Duffy, & Kalivas, 1995).

The increased basal concentration of dopamine in the NAc was found to gradually decline to the level of saline-treated controls following cessation of cocaine treatment (Heidbreder, Thompson, & Shippenberg, 1996; Johnson & Napier, 2000; Segal & Kuczenski, 1992; Weiss et al., 1992). Interestingly, despite the normalisation of basal dopamine levels, after extended periods of withdrawal expression of behavioural sensitisation is associated with increased dopamine transmission and a sensitised locomotor response higher than that found immediately following cessation (Weiss *et al.*, 1992; Heidbreder, Thompson and Shippenberg, 1996). This implies other, long-term adaptive changes occur in response to psychostimulant sensitisation.

A number of signalling molecules are known to be induced by psychostimulants including FosB (Hope *et al.*, 1994), cFos (Robertson et al., 1991) and ERK (Bertran-Gonzalez *et al.*, 2008). In rats cFos is increased in the neurons in the NAc following behavioural sensitisation to cocaine (Crombag et al., 2002).

Further, following sensitisation to cocaine, cFos is increased preferentially in D1 neurons (Young et al. 1991, Bertran-Gonzalez et al. 2008; Guez-Barber et al. 2011) and conditional deletion of cFos in D1 neurons diminished cocaine sensitisation (Zhang et al., 2006).

1.5.3. GABA_A Receptors in behavioural sensitisation to psychostimulants

It is also thought that repeated exposure to cocaine induces changes in GABA systems, resulting in a dysregulation of the neural circuitry mediating behaviour responses to drugs (Koob & Le Moal, 2001; Koob & Volkow, 2010).

Following amphetamine-sensitisation a decrease in GABA_A α 2-subunits is reported within the NAc shell and core (Zhang et al., 2006). Indeed, targeted deletion of the GABA_A α 2 subunit, known to be highly represented within the NAc, blocked the development of behavioural sensitisation to cocaine (Morris et al., 2008). Similarly, selective activation of α 2 receptors within the NAc using intra-NAc infusions of the GABA_A agonist Ro 15-4513, in α 2(H101R) mutant mice, in which the mutation results in a change in efficacy of Ro 15-4513 from a negative allosteric action to a positive allosteric action, was sufficient to induce behavioural sensitisation (Dixon *et al.*, 2010).

Involvement of the GABA_A α 4 subunit in mediating behavioural sensitisation to cocaine has also been suggested. The α 4 subunit is genetically upregulated preferentially in D1 neurons following repeated or high dose cocaine administration (Heiman *et al.*, 2008). Systemic administration of THIP blocks both the induction and expression of behavioural sensitisation to amphetamine (Karler et al., 1997). Constitutive deletion of the α 4 subunit in mice did not affect behavioural sensitisation to cocaine but abolished the ability of systemic or intra-NAc THIP to attenuate sensitisation (Macpherson, 2013). These results indicate that activation of α 4-GABA_ARs is likely to oppose sensitisation to cocaine, though they are not activated under normal conditions.

1.5.4. Psychostimulant induced neuroadaptations

Repeated exposure to psychostimulants results in neuroadaptations of the systems in which they produce their effects which can be maladaptive or homeostatic (Nestler, 1993). Neurophysiological changes are known to underlie behavioural sensitisation to psychostimulants following repeated exposure and

this has been proposed to mediate 'incentive sensitisation' (Robinson & Berridge, 1993; Robinson & Berridge, 2008).

Repeated administration of psychostimulants produces a variety of alterations in dopaminergic neurons, especially within key reward pathway structures such as the NAc and VTA (Nestler, 2005). Transient changes include reduction of inhibitory G protein levels (Nestler *et al.*, 1990; Striplin and Kalivas, 1992), enhanced basal levels of extracellular DA (Kalivas and Duffy, 1993), and enhanced sensitivity of AMPA receptors on VTA DA neurons (White *et al.*, 1995b; Zhang *et al.*, 1997). These transient changes suggest enhanced basal activity of DA neurons.

Intermediately, animals treated repeatedly with psychostimulants displayed enhanced responses of D1 dopamine receptors both pre- and post-synaptically (Henry, Greene and White, 1989; Higashi *et al.*, 1989; Wolf, White and Hu, 1994). Following repeated cocaine administration, a decrease in the density of glutamate but not GABA immunolabeling was observed within the NAc, indicating an increase in excitatory synaptic activity (Meshul *et al.*, 1998). Long-term, intermittent cocaine administration increased spine density on dendrites of MSNs in the NAc (Robinson and Kolb, 1999; Robinson *et al.*, 2001).

Chronic psychostimulant exposure alters expression of many genes (Nestler, 2004). Perhaps most notable is the dramatic increase of the transcription factor protein Δ FosB, thought to act as an important molecular "switch" in the transition from drug abuse to addiction (Nestler, Barrot and Self, 2001; Nestler, 2005). By artificially increasing or decreasing Δ FosB expression Nestler and colleagues (2001, 2004) examined its behavioural effects. Mice with elevated Δ FosB in the NAc exhibited increased sensitivity to cocaine as well as increased self-administration and motivation for cocaine (Kelz *et al.*, 1999). Conversely, blocking the build-up of Δ FosB in mice during a regimen of cocaine exposure reduced these behaviours.

1.5.5. Psychostimulant induced neural ensembles in the reward pathways

The neural substrates of behavioural activation and the perception of reward are often highly coincident or proximate, notably in the Striatum (Robbins and

Everitt, 2002; Koob, 2014). It has been proposed that neuronal 'ensembles' within the NAc may represent various stimulus-action associations which, when activated by conditioned stimuli, compete to control behavioural output (Pennartz, Groenewegen and Lopes da Silva, 1994; Nicola, 2007).

A number of Immediate-Early-Genes (IEGs) genes have been identified which are upregulated following neural activation and therefore provide a histological marker for neural activity (Morgan and Curran, 1991). This has been used to identify spatial patterns of 'neural ensembles' which are activated by environmental stimuli, behavioural tests and drugs (Sheng and Greenberg, 1990; Cruz *et al.*, 2013). Notably, activation of the proto-oncogene cFos has been widely characterised and used to map neural activity (Sheng and Greenberg, 1990).

Histochemical analysis has revealed that cFos is increased in striatal and cortical neurons *in-vivo* following injections of dopamine D1 receptor agonists, D2 receptor antagonists as well as psychostimulants including amphetamine and cocaine (Robertson *et al.*, 1991). Conditional deletion of cFos in D1 expressing neurons ablated a number of persistent neuroadaptations normally induced by cocaine (Zhang *et al.*, 2006). Loss of cFos in D1 neurons attenuated dendritic remodelling and behavioural sensitization after repeated exposure to cocaine; however, it increased persistent memory of cocaine-induced conditioned place preference. This indicates that cFos induced in D1 neurons integrates mechanisms to facilitate both the acquisition and extinction of cocaine-induced persistent changes.

Neural ensembles within the NAc are activated by environmental cues paired with natural rewards or drugs (Pennartz, Groenewegen and Lopes da Silva, 1994; Mattson *et al.*, 2007; Cruz *et al.*, 2013). Cocaine induced cFos is further increased in the NAc following repeated cocaine administration, i.e. behavioural sensitisation, in rats (Crombag *et al.*, 2002). It was subsequently discovered that this effect occurred only when cocaine was administered in the conditioned environment, supporting the idea that ensembles code stimulus-action associations (Mattson *et al.*, 2007). Accordingly, selective inactivation of these neurons with the 'Duan02 inactivation method' attenuated cocaine-induced locomotor sensitisation in animals receiving cocaine in the drug-paired but not

non-paired environment (Koya *et al.*, 2009). These results support a model in which drug-induced dopamine release in the NAc acts to increase the firing of neurons representing stimulus-action associations.

Recently, cFos has been combined with new technologies in transgenic mice, for example tagging cFos with reporters such as GFP which allows electrophysiological recordings from neural ensembles (Koya *et al.*, 2012; Cruz *et al.*, 2013). Electrophysiological recordings, enabled by cFos-GFP mice, reveal that ensembles activated by food-paired environments are found to exhibit increased intrinsic excitability relative to surrounding populations (Ziminski *et al.*, 2017) whereas cocaine induced ensembles exhibit significantly attenuated glutamatergic synaptic strength or 'silent synapses' due to loss of AMPA receptor function.

1.5.6. Psychostimulant modulation of GABA_AR subunit expression

GABAergic mechanisms also appear to play an important role in mediating the physiological and behavioural effects of psychostimulants. Early studies revealed systemic amphetamine administration resulted in reduced extracellular GABA concentration within the VP (Bourdelaïs and Kalivas, 1990). Similarly, it was observed that chronic cocaine administration selectively attenuated GABA_AR function within the striatum (Peris, 1996). Evidence was mixed as other studies have found no effect of psychostimulant administration on either quantity or function of GABA_ARs within the striatum (Jung and Peris, 2001).

By analysing expression of various GABA_A receptor subunits and in specific cell populations psychostimulant effects on the expression of specific GABA_AR isoforms has been revealed. Chronic cocaine treatment was found to induce robust up-regulation of $\alpha 4$ subunit gene expression selectively in D1-MSNs (Heiman *et al.*, 2008). Acute cocaine administration resulted in decreased $\alpha 1$ subunit expression in the striatum when measured 1-hour post-administration (Suzuki *et al.*, 2000). In contrast, when measured following cocaine self-administration, at both 1 day (acute) and 20 days (chronic), $\alpha 1$ subunit mRNAs were up-regulated, whereas $\alpha 4$, $\alpha 6$, $\beta 2$, $\gamma 2$, and δ subunits were downregulated in the VTA (Backes and Hemby, 2003).

In pre-clinical studies quantitative immunohistochemistry of GABA_AR subunit proteins revealed a significant decrease in $\alpha 2$ subunits within the hippocampal dentate gyrus and CA1 regions following chronic cocaine administration (Lilly and Tietz, 2000). Furthermore, following methamphetamine-sensitisation a decrease in GABA_AR $\alpha 2$ was also observed in the NAc shell and core (Zhang et al, 2006). Accordingly, reversal of cocaine-induced behavioural sensitisation by pergolide/ondansetron treatment normalises GABA_AR $\alpha 2$ subunit expression within the NAc (Chen and Olsen, 2007).

As previously discovered in alcoholism, genetic studies have linked the GABRA2 gene with cocaine addiction (Dixon *et al.*, 2010). It has further been proposed that some GABRA2 haplotypes may interact with experience of childhood trauma to influence risk of cocaine dependence (Enoch *et al.*, 2010). A GABRA2 haplotype, which was negatively associated with addiction, was positively associated with resilience to addiction following childhood trauma.

1.5.7. Psychostimulants and conditioned behaviours

It has been proposed that drug-associated cues, environmental or discrete, are able to trigger drug craving and therefore contribute to high rates of relapse in people with (Stewart, de Wit and Eikelboom, 1984; Robinson and Berridge, 1993; Everitt, Dickinson and Robbins, 2001; Crombag *et al.*, 2008). Cocaine-associated cues were able to trigger increased physical arousal and craving for cocaine in abstinent cocaine users (Avants *et al.*, 1995) and neuroimaging studies have demonstrated that exposure to cocaine-related stimuli resulted in an increase in striatal dopamine release (Volkow *et al.*, 2006).

Pre-clinical rodent models have been widely used to establish the role of the mesolimbic dopamine system in development and expression of associative learning. These procedures include cue-induced reinstatement of drug-seeking, conditioned place preference, conditioned reinforcement and second-order schedules of reinforcement (Everitt, Dickinson and Robbins, 2001; Tzschentke, 2007; Crombag *et al.*, 2008). Primarily these models rely on Pavlovian conditioning whereby repeated pairing of an unconditioned stimulus (US), i.e. contextual or discrete cues, with a reward, can result in the US acquiring the motivational properties of the primary reward and become a conditioned

stimulus (CS). Combining these models with genetic and pharmacological manipulation of specific anatomical and molecular targets can elucidate the neurobiological mechanisms by which drug-associated stimuli illicit drug-seeking.

As well as mediating acute behavioural and primary reinforcing effects of drugs of abuse, the NAc is also implicated in the ability of reward-paired environmental cues to motivate drug-seeking behaviour (Everitt and Robbins, 2005). The NAc has been proposed to encode the predictive value of reward associated stimuli and to stimulate specific, goal-directed motor behaviours in. It has been proposed that NAc neurons encode sensory information associated with the consequences of different behaviours and that this activity promotes reward-seeking behaviour. This was supported by the observation that NAc neurons excited and inhibited by cues showed larger firing changes in response to a CS than US, and larger changes when the animal made an operant response to the cue than when the animal failed to respond (Yun *et al.*, 2004). Excitations during operant responding were not modulated by cues, whereas inhibitions during operant responding were larger if the operant response occurred during the reward-associated cue.

1.5.8. GABA_ARs in the NAc mediate cocaine effects on reward-conditioned behaviours

A model has been proposed in which striatal neurons compete for control over basal ganglia output nuclei and are inhibited by GABAergic interneurons and collateral connections between neighbouring MSNs to facilitate action selection by the NAc (Nicola, 2006). Given these findings it is likely that specific GABA_AR isoforms are responsible for modulating the activity of NAc MSNs which underlie behavioural responses to psychostimulants and reward-conditioned cues. GABA_AR subunits that are expressed within the NAc, such as $\alpha 2$ and $\alpha 4$, are therefore targets of interest to modulate these behaviours.

In a conditioned reinforcement (CRf) experiment deletion of the $\alpha 2$ subunit did not modulate instrumental responding for reward-conditioned cues; however, it abolished cocaine potentiation of responding (Dixon *et al.*, 2010). This suggests that $\alpha 2$ -GABA_ARs do not mediate learning of reward-associated cues, but they are necessary for cocaine to facilitate cue-induced behaviours.

In contrast, deletion of the $\alpha 4$ subunit, constitutively or specifically in the NAc, enhanced baseline and cocaine potentiated CRf responding. Further deletion of $\alpha 4$ in D2-, but not D1-, expressing neurons alone was sufficient to produce these effects. Thus, tonic inhibition of D2 MSNs in the NAc by $\alpha 4$ -GABA_ARs opposes conditioned reinforcement and cocaine's ability to facilitate cue-induced behaviours.

1.6. Investigating the role of $\alpha 4$ -GABA_ARs in mediating addiction-related behaviours

As outlined above, $\alpha 4\beta\delta$ GABA_AR subtypes are the most rapidly regulated in plastic mechanisms triggered by high-dose alcohol or chronic exposure to alcohol in rats (Liang *et al.*, 2007), and within the NAc they are functionally associated with alcohol consumption and reinforcement (Boyle *et al.*, 1993; Rewal *et al.*, 2009, 2012). These receptors are unlikely to be directly sensitive to ecologically valid doses of ethanol, although it may modulate them indirectly via neurosteroids. Alternatively, it is possible that $\alpha 4$ -GABA_ARs play a more general role in reward learning and motivation.

While the role of $\alpha 4\beta\delta$ GABA_ARs in alcohol related behaviour is highly studied, less is known about the possible involvement of $\alpha 4$ -GABA_ARs in mediating the rewarding and reinforcing of drugs of abuse other than alcohol. Notably, within the striatum $\alpha 4$ -GABA_ARs are most highly expressed within the NAc, where we have demonstrated they mediate a tonic form of inhibition that acts to control the excitability of MSNs (Maguire *et al.*, 2014).

Prior to the experiments presented in this thesis, investigators in our laboratory conducted a series of studies using genetic manipulations of the $\alpha 4$ subunit in mice to elucidate the role of $\alpha 4$ -GABA_ARs in the acute effects of cocaine and cocaine conditioned behaviours (Macpherson, 2013), summarised below.

Firstly, constitutive deletion of the $\alpha 4$ subunit did not affect baseline locomotor behaviour or its potentiation by cocaine; however, it abolished the ability of systemic or intra-NAc THIP to block cocaine potentiated locomotor activity. Secondly, we investigated the role of $\alpha 4$ -GABA_ARs in natural- and cocaine-conditioned behaviours using tests of behavioural sensitisation, cocaine-CPP with and without cocaine priming, and conditioned reinforcement (CRf) to natural

rewards with and without cocaine priming. Deletion of GABA_AR α 4-subunits did not alter the augmentation of locomotor activity observed following repeated, intermittent cocaine, indicating that unlike α 2-GABA_ARs, α 4-GABA_ARs are not involved in the development of behavioural sensitisation to cocaine. As with locomotor activity following acute cocaine, systemic THIP was able to reduce the sensitised increase in locomotor activity in wild type but not α 4 knockout mice. Together these results indicate α 4-GABA_AR-mediated inhibition of NAc MSNs is able to attenuate the ability of cocaine to potentiate locomotor activity however under normal physiological conditions, there exists little tonic inhibition. Whilst constitutive deletion of the α 4 subunit had no effect on CPP we demonstrated that a conditional deletion of α 4 specifically on D1-, but not D2-, MSNs was sufficient to increase CPP and cocaine's ability to potentiate CPP. Conversely, both constitutive, NAc specific and D2-, but not D1-, specific deletion of the α 4 subunit increased responding for conditioned reinforcers and further increased cocaine's ability to potentiate responding in the CRf experiment.

These experiments clearly demonstrate that α 4-GABA_ARs modulate cocaine induced locomotor activity and conditioned behaviour. They also identify a dissociation between conditioning effects of α 4-GABA_ARs on D1- vs D2-MSNs whereby they modulate environmental conditioning and learning conditioning to discrete cues respectively. However, many questions remain to be answered. Firstly, we may hypothesize that whilst constitutive deletion of α 4-GABA_ARs had no effect, D1- or D2-MSN specific deletion may affect baseline locomotor behaviour or its potentiation by cocaine. This is a very important consideration as it may underlie the expression of other behavioural differences in CPP and CRf experiments. Further, we wished to examine the roles that α 4-GABA_ARs on D1- or D2-MSN may have on the development of behavioural sensitisation to cocaine. Given that chronic cocaine treatment was found to induce robust up-regulation of α 4 subunit gene expression selectively in D1-MSNs (Heiman et al., 2008) we may expect altered behavioural sensitisation when this mechanism is removed. Finally, results from our CRf experiments indicate that α 4-GABA_ARs on D2-MSNs oppose instrumental responding for reward-associated-cues, i.e. secondary reinforcers. We therefore wished to examine whether α 4-GABA_ARs on D2-MSNs also influenced responding and motivation for primary reinforcers themselves. If correlated this may underlie responding for secondary reinforcers.

We tested these hypotheses in well-established behavioural tests, including behavioural sensitisation, and operant conditioning under fixed and progressive ratio schedules of reinforcement.

1.7. Aims and Structure of the thesis

Chapter 1

General Introduction.

Chapter 2

We have generated several transgenic mouse lines in which $\alpha 4$ has been deleted either constitutively or within specific neural populations. In chapter 2 we aim to confirm the deletion of $\alpha 4$ has occurred in each line as expected. We used mice in which $\alpha 4$ had been deleted constitutively ($\alpha 4$ -KO) or conditionally in D1 or D2 type dopamine receptor expressing neurons via Cre-lox recombination (Gong *et al.*, 2007). We performed PCR with primers targeting each transgene gene to genotype these animals. We then confirmed the deletion using quantitative rt-PCR to and in-situ-hybridisation to compare *gabra4* mRNA levels in brain sections from each genotype. Using sections from conditional knockouts we performed a multi-probe fluorescent in-situ-hybridization method (RNAScope) to confirm in which cell types Cre-recombinase was expressed and that deletion of $\alpha 4$ occurred in these populations specifically. We also generated an Adeno Associated Virus (AAV) carrying Cre-recombinase to knockdown $\alpha 4$ locally by infusing it into in specific brain regions of 'floxed'- $\alpha 4$ mice. To verify the efficacy of this virus we infused it into the brains of those mice then sectioned their brains for immunohistochemical and rt-PCR analysis. Characterising these lines verified our transgenic manipulations and allows us to attribute behavioural differences to the activity of $\alpha 4$ -subunits in further experiments.

Chapter 3

Chapter 3 explores the role of GABA_ARs containing $\alpha 4$ -subunits in controlling binge-like alcohol consumption. We used a well-established experimental protocol called 'Drinking in the Dark' (DID) to monitor their voluntary alcohol consumption (Wise, 1973). In DID experiments mice typically consume relatively large volumes of alcohol in a short time making it a good model for binge

drinking (Crabbe, Harris and Koob, 2011). We compared constitutive $\alpha 4$ -KO mice with wild-type littermates in a standard DID experiment to assess whether deletion of $\alpha 4$ would alter ethanol consumption. Following this we attempted to isolate the effects of this manipulation to the NAc where $\alpha 4$ is highly expressed. Using 'floxed' $\alpha 4$ mice we targeted the NAc with a virus transfecting Cre-recombinase to delete $\alpha 4$ there specifically and observed the effect on DID. We also measured DID following pharmacological activation of $\alpha 4$ -GABA_ARs by the drug THIP both systemically at various doses and delivered directly to the NAc. Finally, we compared DID in mice where $\alpha 4$ was deleted conditionally in D1 or D2 type neurons to see if $\alpha 4$ driven effects were mediated by either population. The aim of this chapter is to determine whether $\alpha 4$ -GABA_AR activity in the NAc controls ethanol consumption and identify which $\alpha 4$ -expressing neural populations do so. This may provide pre-clinical evidence that $\alpha 4$ -GABA_ARs are a target for therapies to treat alcoholism.

Chapter 4

Previously conditional deletion of $\alpha 4$ in D1 or D2 type neurons has had different effects on cocaine conditioned behaviour (Maguire *et al.*, 2014). In chapter 4 we expand on these studies by exploring the role of $\alpha 4$ -GABA_AR in controlling locomotor activity and its potentiation by acute cocaine. We performed a cocaine dose response in D1 or D2 $\alpha 4$ conditional knockout mice to assess whether deletion of these receptors in either population alone altered the locomotor response to cocaine at various doses. This will also allow consideration for other behaviours that could be affected by changes in locomotor activity. Conditional deletion of $\alpha 4$ in D1 or D2 type neurons also differently altered responses to reward conditioned cues and their potentiation by cocaine (Macpherson *et al.*, 2016). We therefore examined the effect of $\alpha 4$ -subunits in D1 or D2 type neurons on instrumental responding for primary rewards to identify whether this may underlie motivation for cues or if the effect of $\alpha 4$ on responding for cues was distinct from motivation for the primary reward. We trained D1 and D2 $\alpha 4$ conditional knockout mice to lever-press for sucrose rewards under fixed and progressive ratio conditions and compared their responding. This informs previous findings and explores differential roles of D1 and D2 neurons in responding for primary vs secondary rewards.

Chapter 5

$\alpha 2$ -containing GABA_ARs have previously been shown to be crucial for behavioural sensitisation to cocaine (Dixon et al., 2010) whilst constitutive deletion of $\alpha 4$ did had no effect (Macpherson, 2013). The aim of chapter 5 was to investigate whether populations of $\alpha 4$ expressing neurons mediate behavioural sensitisation to cocaine. Constitutive $\alpha 4$ -KO mice, D1 and D2 $\alpha 4$ conditional knockout mice and their wild-type littermates were given repeated, intermittent cocaine or saline over 10 sessions. Conditioned locomotor response to cocaine was explored by testing for conditioned activity following saline in the drug-paired context. Following these experiments mice were immediately sacrificed and brains were used in immunohistochemistry experiments to examine expression of the neural activity marker cFos throughout the striatum. We compared cFos expression in the Core and Shell sub-regions of the NAc which are known to play different roles mediating the effects of psychostimulants (Ito, Robbins and Everitt, 2004). We also used RNAScope methods on the brains of $\alpha 4$ -KO mice to examine whether effects of cocaine sensitisation on cFos differed in D1 and D2 neural populations in these sub-regions. These experiments provide novel data into a possible role of neural populations modulated by extrasynaptic GABA_ARs in mediating behavioural responses to repeated cocaine administration.

Chapter 6

General Discussion.

Chapter 2

Characterising transgenic-mice and viral-vectors used to manipulate $\alpha 4$ -containing GABA_A receptors

2.1. Introduction

2.1.1. $\alpha 4$ -GABA_AR subunits in D1 and D2 striatal neurons

The GABA_AR $\alpha 4$ subunit is distributed throughout the thalamus, NAc, hippocampus, neocortex and caudate-putamen (Wisden et al., 1992; Pirker et al., 2000; Schwarzer et al., 2001). Within the striatum $\alpha 4$ is most highly expressed in the NAc. As GABA_A receptor sub-types in the striatum and NAc have been implicated in addiction and drug abuse by numerous studies (Macpherson, 2013; Maguire et al., 2014; Stephens et al., 2017) we wished to investigate the role of $\alpha 4$ -containing receptors ($\alpha 4$ -GABA_ARs) in these disorders. In particular we wished to investigate the behavioural effects of manipulating $\alpha 4$ -GABA_ARs in distinct regions and neural populations within the striatum.

The majority of the striatum (~95%) is made up of GABAergic Medium Spiny Neurons (MSNs) so named due to their medium size and extensive dendritic trees (Kemp and Powell, 1971). MSNs have also been characterised electrophysiologically by their hyperpolarised resting membrane potential and low input resistance (C. Wilson and Kawaguchi, 1996). Historically MSNs were characterised by their morphology and divided into two populations, known as the direct and indirect pathways, based on their axonal projection targets (Bolam et al., 2000).

The direct pathway originates in striatonigral neurons which form monosynaptic inhibitory connections with SNr/GPi neurons, suppressing inhibition of the thalamus, and ultimately disinhibiting selected behaviours (Vincent et al., 1982; Christensson-Nylander et al., 1986; Chevalier and Deniau, 1990). The indirect pathway originates in striatopallidal neurons which project to the GPe and onto the SNr/GPi complex via a polysynaptic disinhibitory connection, and an indirect GPe-STN-GPi connection, ultimately inhibiting the thalamus and suppressing

selected behaviours (Beckstead and Kersey, 1985; Gerfen *et al.*, 1990; Albin, Young and Penney, 1995; Cohen and Frank, 2009).

MSNs of the direct and indirect pathway have different molecular profiles so they were initially identified as distinct subtypes using their releasable neuropeptides as cell-type specific markers (Gerfen and Scott Young, 1988; Le Moine and Bloch, 1995). Immunohistochemistry identified populations of striatal neurons expressing the neuropeptides dynorphin and substance P and others expressing enkephalin (Beckstead and Kersey, 1985; Christensson-Nylander *et al.*, 1986).

Fluorescent retrograde tracing in combination with in-situ hybridisation were used to demonstrate that striatonigral neurons co-express neuropeptides substance P and dynorphin whilst striatopallidial neurons express enkephalin; this provided the first markers to define these distinct but co-localised neuronal populations (Gerfen and Scott Young, 1988). Subsequent experiments in 6-hydroxydopamine (6-OHDA) lesioned mice used D1 and D2 dopamine receptor specific agonists (SKF-38393 and quinpirole) in combination with retrograde tracing and mRNA profiling to demonstrate that dynorphin/substance P or enkephalin expression in MSNs were differentially associated with D1R or D2R expression respectively (Gerfen *et al.*, 1990).

MSNs can be therefore divided into two subtypes; D1 and D2 MSNs based on their expression of D1 or D2 class dopamine receptors. Previously D1 and D2 receptors had been presumed to co-localize but this and further studies using sensitive cDNA probes (Le Moine and Bloch, 1995) strongly indicated that D1Rs and D2Rs are expressed in distinct MSN subtypes which are segregated into discrete pathways. A large body of research has elucidated distinct and often opposing functions of these two populations, sometimes referred to as the go/no-go pathways due to their roles in action initiation/inhibition (reviewed, Surmeier, 2013).

The segregation of D1R and D2R expression to the direct and indirect pathways respectively is well established in the dorsal striatum however recently evidence indicates that this is not so in the ventral striatum. Kupchik and colleagues used a Cre-dependent ('floxed') channelrhodopsin (ChR2) viral vector to optogenetically activate D1 or D2 neurons in the NAc core of Cre-expressing

transgenic mice (Kupchik *et al.*, 2015). They recorded GABAergic IPSCs from the Ventral Pallidum (VP) following optogenetic stimulation of either population and found that up to 50% of dorsal VP neurons are innervated by both D1 and D2 MSN afferents. We must therefore exercise caution in referring to D1 and D2 populations as the 'direct' or 'indirect' pathways within the ventral striatum.

As these MSN subtypes heterogeneously populate the same regions dissecting the neurocircuitry of the striatum requires high-resolution techniques to investigate and map its cytoarchitecture. It is important to target discrete neural populations within defined regions that may be anatomically but not functionally overlapping which has proved difficult using traditional methods. Traditional histochemistry and mechanically injected anterograde/retrograde tracers such as horseradish peroxidase (HRP), fluorescent dyes and labelled plant lectins are not heritable or specific to genetically defined neurons (Feng *et al.*, 2000). While immunohistochemistry allows us to label neuronal subtypes it is based on markers which usually must first be genetically defined. New methods that genetically target expression of 'reporter genes' are able to overcome these problems and achieve greater anatomical resolution.

Using recombinant DNA techniques genetic constructs can be placed under the control of various cis-regulatory elements, these can encode molecules such as beta-galactosidase (LacZ), which allow easy identification of the cells in which they are expressed, i.e. 'reporter genes' (Forss-Petter *et al.*, 1990). This type of labelling is more reliable than immunohistochemistry which requires markers that are based on correlative data rather than being genetically hard-coded. The discovery and development of green fluorescent protein (GFP) as a marker for gene expression has rapidly become a staple technique for mapping neural populations as it allows stable reporter expression with minimal perturbation to the cell (Chalfie *et al.*, 1994).

Transgenic mice expressing several GFP variants with different optical properties (collectively named XFP) under different promoters to allow imaging of neuronal subsets based on their expression patterns (Feng *et al.*, 2000). This was particularly useful as it allows the imaging of multiple distinct populations, concurrently, based on optical properties of the fluorescent marker expressed. The method was not entirely reliable however as marker expression was found

to vary between founder lines. Variation can be caused by variable regulatory elements in the integration site or other effects such as silencing due to nearby chromatin known as position effect variegation (Festenstein *et al.*, 1996).

In a refinement of this transgenic method GFP was expressed under the control of more regulatory elements. This was achieved using Bacterial Artificial Chromosomes (BACs) as vectors for these reporters (Gong *et al.*, 2003). BACs are highly useful as they can carry large regulatory sequences and buffer transgenes from local regulation. This has given rise to over 250 driver lines including 20 lines that drove expression within the striatum. Importantly, GFP has been expressed under the *Drd1* or *Drd2* dopamine receptor. BAC mice were also generated carrying a red fluorescent protein under the *Drd1* promoter (*Drd1a*-tdTomato) and crossed with BAC-*Drd2*-eGFP to allow simultaneous visualization of D1 and D2 populations (Shuen *et al.*, 2008). Characterisation of these mice conclusively demonstrated that D1 and D2 receptors are expressed dichotomously in two distinct MSN populations, less than 1% of cells showed detectable expression of both fluorophores.

2.1.2. Using conditional knock-outs to functionally differentiate D1 and D2 MSNs

The bacteriophage P1 recombinase 'Cre' can be used to mediate recombination between short sequences called 'loxP' sites (Hoess and Abremski, 1985, see Chapter 1). The 'Cre-lox' recombination system has been further developed for use in mammals (Sauer and Henderson, 1988). Transgenic mice expressing Cre can be crossed with mice carrying a sequence which have been flanked by loxP sites via homologous recombination (i.e. 'floxed') resulting in deletion of that intervening sequence in the offspring (Lakso *et al.*, 1992). This system is used in mice carrying a 'floxed' gene of interest so that it will be knocked out in a compartmentalised, cell-specific manner which is determined by the promoter under which Cre is regulated (Gu *et al.*, 1994). Crucially this allows selective knockout of genes in defined cell types, such as D1 and D2 MSNs, so that their component function can be examined separately.

As was previously done with GFP reporters a library of BAC-Cre mice have been generated where Cre is expressed in genetically defined neuron subsets in over

250 driver lines (Gong *et al.*, 2007). These have successfully been used to knock-out receptors and signalling molecules significant to drug seeking behaviour in D1 and D2 MSN populations with behavioural consequences. Conditional KO of DARP-32 in D1 MSNs resulted in hypoactivity and reduced psychostimulant response and in D2 neurons had the reverse effect; hyperactivity and enhanced psychostimulant response (Bateup *et al.*, 2010). Conditional knock-out of the BDNF receptor Trkb in D1 neurons decreased cocaine CPP but in D2 neurons increased CPP. These results identify important mediators of plasticity in MSNs downstream of D1 and D2 neurotransmitter receptors which are of clinical significance. They also support findings in knock-out mice that D1 or D2 expressing populations have different and opposing effects on movement and drug responses (see Chapter 1). Notably, cholinergic interneurons also express D2 dopamine receptors and therefore must be considered when interpreting experiments in which transgenes, such as Cre, are controlled by the *Drd2* promoter, as in our experiments (Dawson, Dawson and Wamsley, 1990).

We have therefore generated dopamine D1 and D2 neuron specific GABA_A $\alpha 4$ -subunit knockout mice and respective wildtypes in order to study the role of $\alpha 4$ -GABA_ARs in behaviours relating to drugs of abuse. We achieved this by breeding 'floxed' $\alpha 4$ mice with BAC mice (Gong *et al.*, 2007, described above) hemizygotously expressing Cre in D1 or D2 neurons (Maguire *et al.*, 2014; Macpherson *et al.*, 2016). In a cocaine-conditioned place preference (CPP) experiment constitutive $\alpha 4$ knockout mice did not differ from WT mice however conditional knock-out of $\alpha 4$ in D1 neurons increased CPP whilst both constitutive and D2 neuron specific knockout enhanced conditioned reinforcement (Macpherson, 2013; Maguire *et al.*, 2014).

Whilst these experiments indicate that deletion of $\alpha 4$ in D1 or D2 neurons is having distinct behavioural outcomes we have yet to fully characterise these conditional D1/D2 $\alpha 4$ knockout mice. Here we use several genetic and immunological methods to demonstrate that the Cre transgene is being expressed in the correct D1/D2 MSN populations and that it is functionally deleting the $\alpha 4$ subunit.

2.1.3. Using viral vectors to achieve spatial resolution

Transfection of genetic constructs by viral vectors provides the best combination of temporal and spatial resolution possible. This has the advantages of anatomical specificity and avoids developmental compensation observed in transgenic mice (Brickley *et al.*, 2001). Disadvantages are poorer stability and efficiency relative to traditional transgenics and that constructs must not exceed the vectors capacity which varies from ~10kb to ~4.5kb depending on the type of virus (Betley and Sternson, 2011).

RNA interference is an evolutionarily conserved gene silencing mechanism which has been co-opted as an experimental tool. The introduction of double stranded RNA sequences such as short hairpin RNA (shRNA) can result in cleavage of messenger RNA to which they are complementary and thereby 'knock-down' of genes of interest (Fire *et al.*, 1998). Both 'Cre-lox' and inducible systems can be used to express RNAi with genetic, spatial and temporal control and viral transfection methods can be combined simply and effectively with RNAi as the small RNA constructs are easily packed into viral vectors (Ventura *et al.*, 2004; Kappel *et al.*, 2007). This has enabled RNAi knock-down of genes in vivo in the mammalian brain. RNAi was used to knock down tyrosine hydroxylase (TH), required for dopamine production, in midbrain neurons which resulted in locomotor deficits and reduced response to psychostimulants (Hommel *et al.*, 2003).

Virally mediated RNAi has also been used to knockdown $\alpha 4$ -GABA_ARs with consequences on drug-related behaviour. Knockdown of $\alpha 4$ in the NAc, by RNAi constructs delivered in a lentivirus, reduced voluntary self-administration of alcohol in rats (Rewal *et al.*, 2009, 2012). Further, knockdown of $\alpha 4$ in the NAc using the same method in mice reduced conditioned reinforcement (CRf) and its potentiation by cocaine (Macpherson *et al.*, 2016). Such lentiviral vectors are only transiently expressed, so RNAi knockdown of gene expression occurred during a small time-window (~5 days). This method is not suitable for experiments which require the observation of behavioural consequences of gene knockdown over longer periods. We therefore wished to develop a permanently expressed Adeno-Associated-Virus (AAV) to knockdown $\alpha 4$ in our experiments.

One major advantage of viral vectors is that they can be readily combined with any technique that makes use of small transgenes to augment them with regional specificity. Soon after development of Cre-reporter mice (Tsien *et al.*, 1996) viral expression of Cre could be used to generate knockouts with both spatial and temporal resolution in mice with 'floxed' gene-of-interest (Kaspar *et al.*, 2002). This method has been used to knockout $\alpha 4$ in the thalamus of 'floxed' $\alpha 4$ mice which attenuated fear-conditioning behaviour (Paydar *et al.*, 2014). Here we generated a similar AAV vector carrying the Cre transgene and demonstrate its ability to knockout $\alpha 4$ the NAc of 'floxed' $\alpha 4$ mice.

2.2. Methods

2.2.1. Animals

2.2.1.1. $\alpha 4$ -WT/Het/KO Mice

Constitutive $\alpha 4$ -subunit knockout mice were produced at Sussex University. "Floxed" $\alpha 4$ -subunit homozygous mice (strain name; B6.129-Gabra4^{tm1.2Geh/J}, supplied by The Jackson Laboratory, ME, USA), were crossed with Cre-recombinase expressing hemizygous transgenic mice (strain name; B6.FVB-Tg (Ela-cre)C5379Lmgd/J, supplied by The Jackson Laboratory, ME, USA). Offspring were genotyped and putative Gabra4 heterozygous mice (carrying the CRE transgene (~50% of offspring)) were bred together to generate homozygous knockout, heterozygous (used for breeding) and wildtype littermates (Figure 2.1).

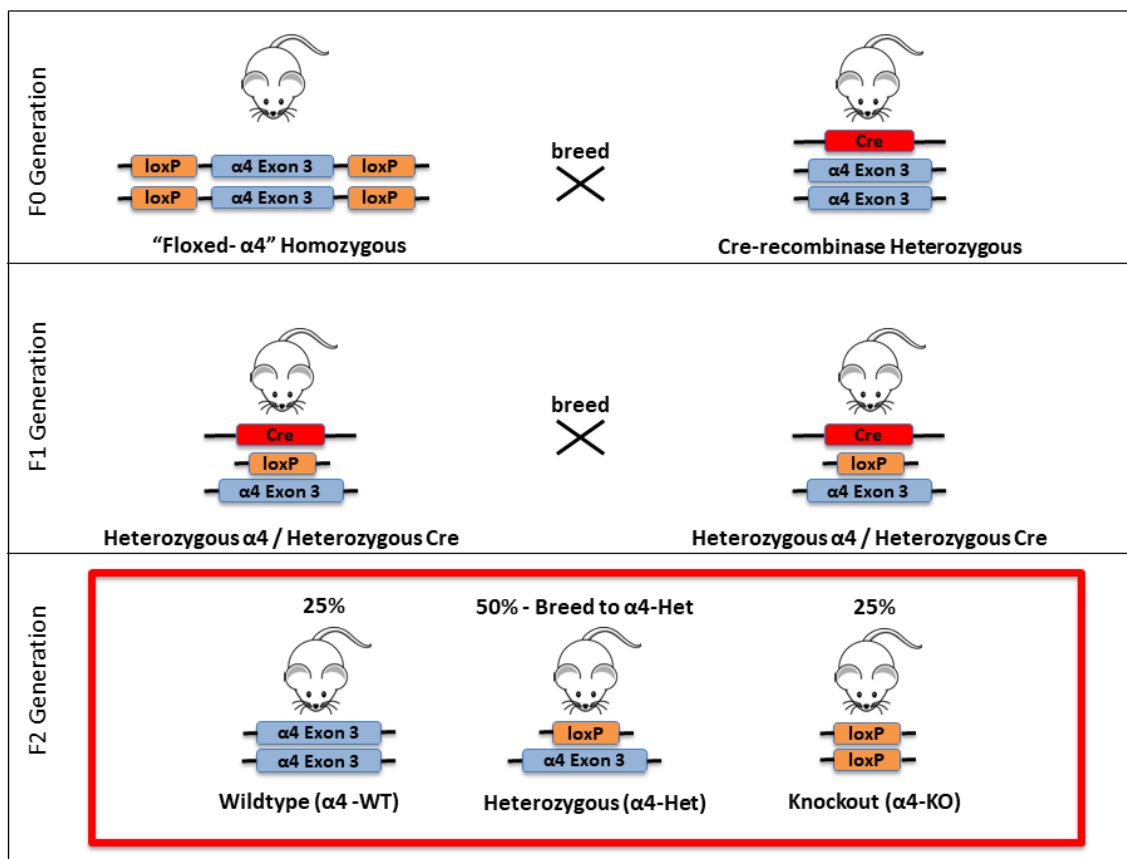
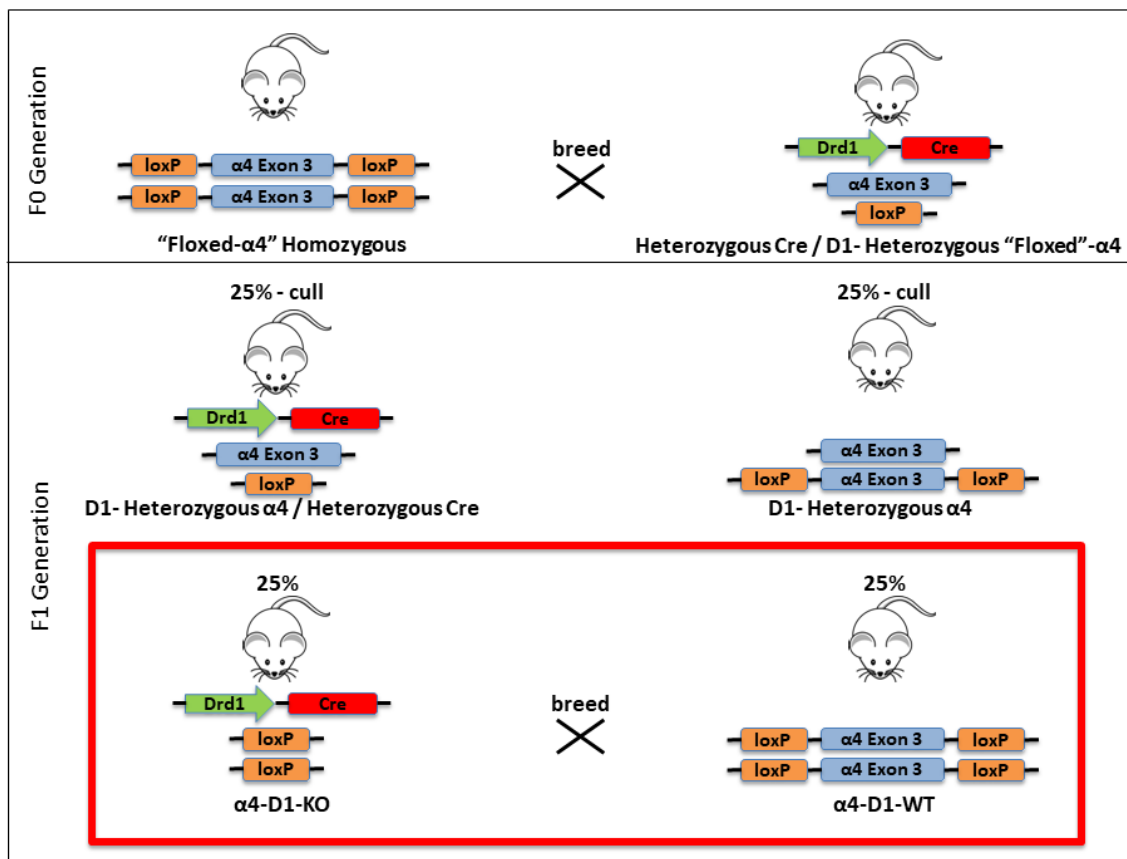
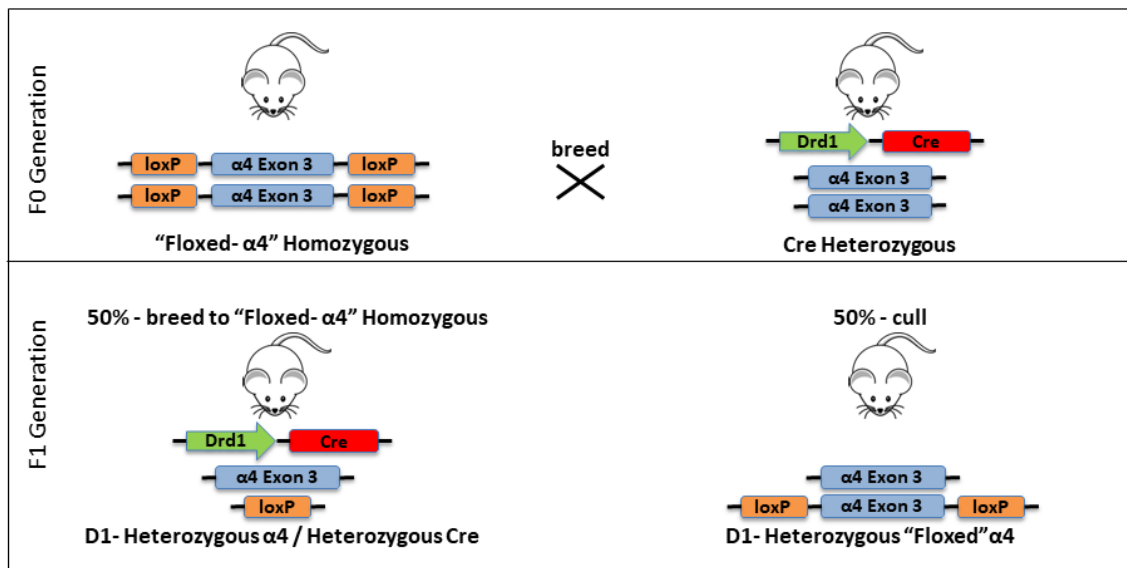


Figure 2.1. Production of $\alpha 4$ -WT, $\alpha 4$ -Het, and $\alpha 4$ -WT mice. (F0) 'Floxed'- $\alpha 4$ homozygous mice were bred with Cre-recombinase expressing heterozygous mice. (F1) offspring were heterozygous for the $\alpha 4$ allele, which were bred to create (F2) offspring in approximate ratios, 25% $\alpha 4$ -WT, 50% $\alpha 4$ -Het, 25% $\alpha 4$ -KO.

2.2.1.2. $\alpha 4$ -D1/D2-KO Mice

Conditional dopamine D1/D2 expressing neuron specific $\alpha 4$ -subunit knockout mice were created by crossing 'Floxed' Gabra4 homozygous transgenic mice (strain name; B6.129-Gabra4tm1.2Geh/J, supplied by The Jackson Laboratory, ME, USA) with either dopamine receptor D1 or D2 neuron specific Cre-recombinase hemizygous transgenic mice (strain name; $\alpha 4$ D1-/- = B6.FVB(Cg)-Tg(Drd1a-cre)EY217Gsat/Mmucd, $\alpha 4$ D2-/- = B6.FVB(Cg)-Tg(Drd2-cre)ER44Gsat/Mmucd, supplied by Mutant Mouse Regional Resource Centers (MMRRC), ME, USA). Breeding was conducted as described in Figure 2.2.



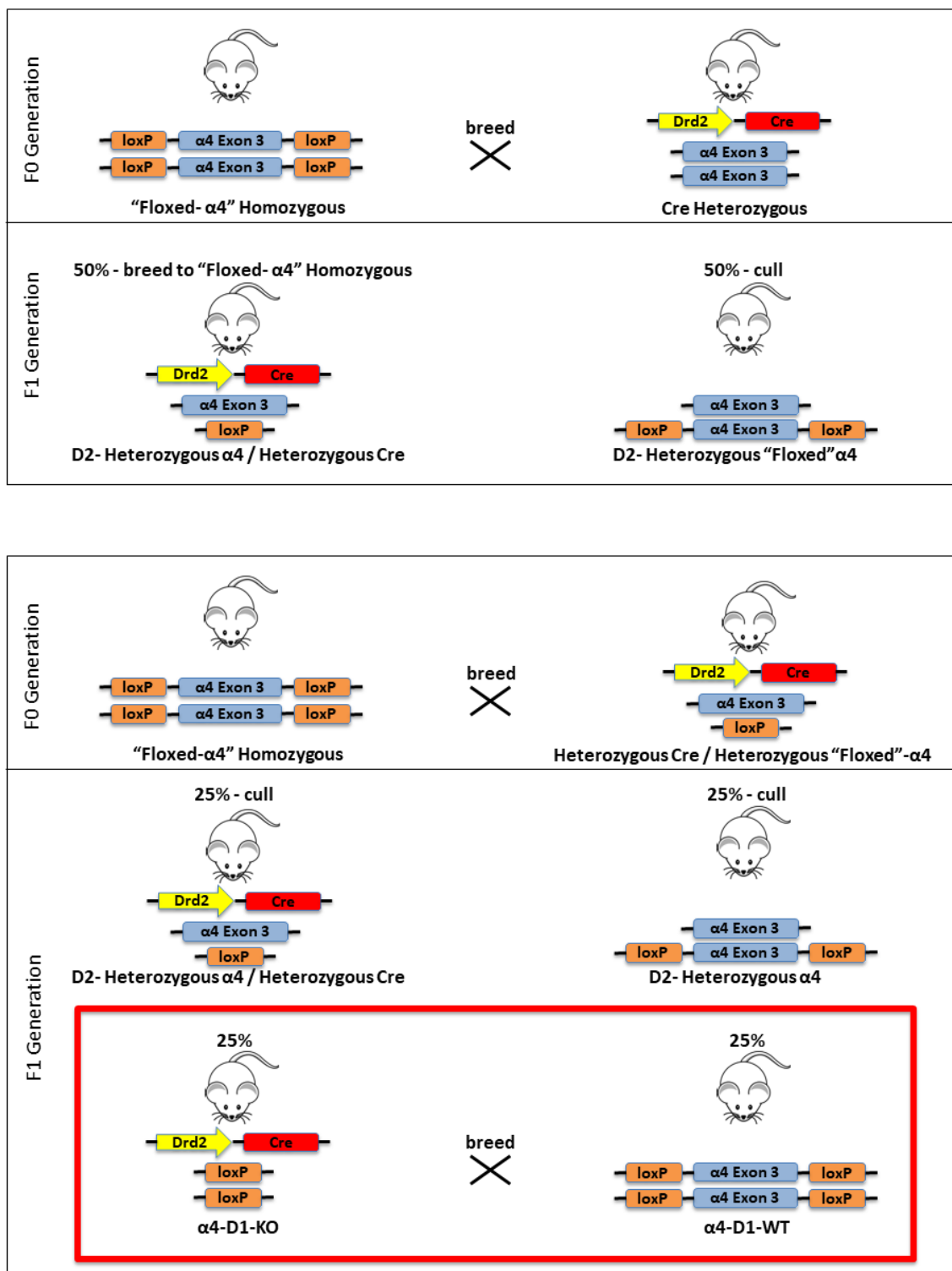


Fig 2.2. Production of D1-neuron specific $\alpha 4$ -subunit wildtype ($\alpha 4$ -D1-WT), and knockout ($\alpha 4$ -D1-KO) mice. **Step 1:** (F0) 'Floxed'- $\alpha 4$ homozygous mice were bred with D1-expressing neuron specific Cre-recombinase expressing heterozygous mice. **Step 2:** (F0) offspring heterozygous for the 'Floxed'- $\alpha 4$

allele, and heterozygous for D1-CRE were bred to create (F1) offspring of approximately; 25% heterozygous for the 'Floxed'- $\alpha 4$ allele, and heterozygous for D1-CRE, 25% heterozygous for the 'Floxed'- $\alpha 4$ allele, and homozygous for No-D1-CRE, 25% 25% homozygous for the 'Floxed'- $\alpha 4$ allele/heterozygous for D1-CRE and 25% homozygous 'Floxed'- $\alpha 4$ allele/homozygous for No-D1-CRE .

Step 3: F(2) offspring homozygous for 'Floxed'- $\alpha 4$ allele/homozygous no-D1-CRE mice and homozygous 'Floxed'- $\alpha 4$ allele/heterozygous D1-CRE mice were used to breed the experimental $\alpha 4$ -D1-WT (50%) and $\alpha 4$ -D1-KO (50%) experimental mice, respectively. The same strategy was used using D2-CRE mice.

2.2.1.3. Animal Husbandry

Male and female GABA_AR $\alpha 4$ -WT, $\alpha 4$ -Het, $\alpha 4$ -KO and dopamine D1- or D2-expressing neuron specific $\alpha 4$ wildtype ($\alpha 4$ -D1-WT/ $\alpha 4$ -D2-WT) and knockout ($\alpha 4$ -D1-KO/ $\alpha 4$ -D2-KO) mice on a C57Bl/6J background strain, weighing between 20-30g, were housed in groups of 2-3, or separately for those undergoing surgery, with food and water available ad libitum. A 12hr light/dark cycle was used (lights on at 7:00 A.M.) with holding room temperature maintained at 21 \pm 2°C and humidity 50 \pm 5%. All injections, infusions and behavioural testing were performed between 2:00 P.M. and 5:00 P.M. All procedures were conducted in accordance to Animals (Scientific Procedures) Act 1986, following ethical review by the University of Sussex Ethical Review Committee.

2.2.2. Genotyping

2.2.2.1. DNA Extraction

Mouse ear punches were collected, and DNA extracted by digestion in a 20 μ l solution of a 1mg/ml proteinase K solution (50mg/ml; Roche Products Ltd., UK) and 20mM Tris HCl (Sigma-Aldrich, Dorset, UK) and 10mM EDTA (Sigma-Aldrich, Dorset, UK) lysis buffer. Solutions were overlaid with two drops of purified mineral oil (Sigma-Aldrich, Dorset, UK), then incubated at 55°C for 2 hours, then heated to 95°C for 15 minutes in a thermocycling PCR machine (G-Storm GS1, GRI Ltd., Somerset, UK). Extracted DNA samples were diluted to 100 μ l with purified PCR water, with gentle mixing.

2.2.2.2. Primers

GABA_AR α 4-subunit PCR primer sequences were used from those presented in the supplementary text of (Chandra et al, 2006). Forward and reverse cDNA primers were designed to target and replicate a sequence within the wildtype *gabra4* gene and the shortened gene with a deletion of exon 3 in the α 4-subunit knockout mouse. The wildtype primers consisted of a 156bp product (forward primer, AAGATCACCAAGCCAACAGG; reverse primer, TCTTTGGGGAGTTGAGGATG) containing the primary loxP site in the "floxed" mice, and part of the conserved region. The knockout primers consisted of a 241bp product (forward primer, AAGATCACCAAGCCAACAGG; reverse primer, TGCACACTGTAATCCCATC), which flanked the primary and secondary loxP sites either side of exon 3 of the *gabra4* gene.

Forward and reverse cDNA primers were designed to target and replicate a sequence contained within the integrated Cre recombinase transgene. The Cre primer consisted of a 102bp product (forward primer; GCGGTCTGGCAGTAAACTATC, reverse primer; GTGAAACAGCATTGCTGTCACTT).

2.2.2.3. PCR

For each reaction, 0.5 μ l of extracted DNA was mixed into a solution of 0.5 μ l of both forward and reverse primers (25 μ M) and 23.5 μ l of Megamix-Blue (Microzone Ltd., Haywards Heath, UK). Solutions were overlayed with two drops of purified mineral oil, then incubated at 95°C for 5 minutes, followed by 35 cycles of the following; 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, in a thermocycling PCR machine. Finally, PCR samples were held at 72°C for 10 minutes.

2.2.2.4. Gel Electrophoresis and DNA Detection

Following PCR amplification of the targeted DNA, samples were electrophoretically separated on a 1.5% agarose (AGTC Bioproducts Ltd., Leicestershire, UK) gel containing 0.004% ethidium bromide (50mg/ml solution; Sigma-Aldrich, Dorset, UK) in 1% TAE buffer (242g/L tris base (Sigma-Aldrich, Dorset, UK), 57.1ml/L acetic acid (Sigma-Aldrich, Dorset, UK), 0.5M EDTA (14.62g of EDTA (Sigma-Aldrich, Dorset, UK) in 100ml/L dH₂O). Gels placed within a horizontal electrophoresis tank connected to a power supply (BioRad

Laboratories Inc., Hercules, CA, USA) were run at 120v for approximately 30 minutes, and then observed under UV light for the presence of the wildtype and knockout primers (Figure 2.3).

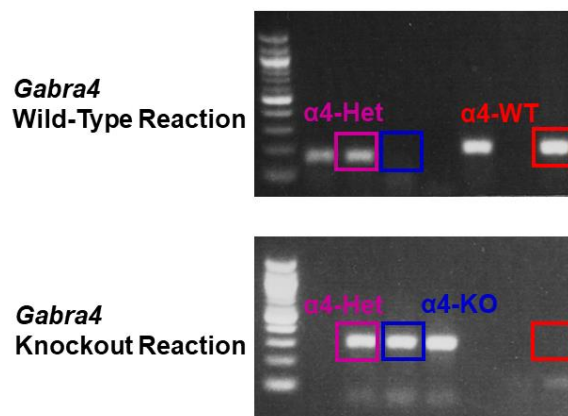


Figure 2.3. Genotyping of GABA_AR α 4-subunit wildtype and knockout mice requires two reactions per mouse. The first reaction contains the wildtype primers, and second reaction contains the knockout primers for detection of the *gabra4* gene. The presence of a band in the wildtype reaction, but not the knockout reaction indicates a wildtype mouse. A band in the knockout reaction, but not the wildtype reaction indicates a knockout mouse. A band in both reactions indicates a mouse heterozygous for both the wildtype and knockout gene.

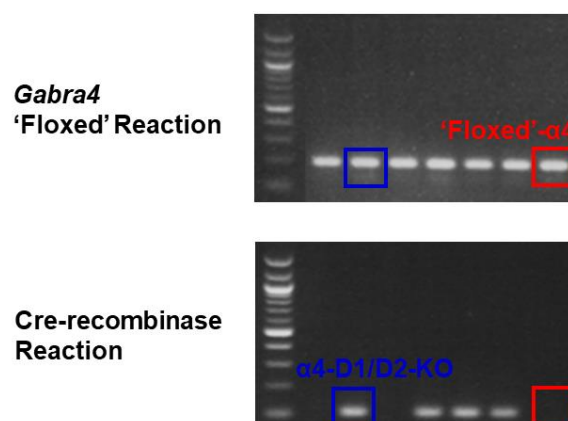


Figure 2.4. The first reaction contains the wildtype primers, which detect the 'Floxed'- α 4 allele. Genotyping of dopamine D1/D2-expressing neuron specific GABA_A α 4-subunit wildtype and knockout mice requires a reaction for the detection of Cre. The absence of a Cre band indicates a wildtype mouse, whilst the presence of a Cre band indicates a knockout mouse.

2.2.3. Quantitative reverse transcription PCR (qRT-PCR) mRNA analysis

2.2.3.1 Preparation of tissue lysates and phase separation

Constitutive GABA_A α 4-subunit wildtype, heterozygous and knockout mice brains were dissected, and tissue samples collected from the nucleus accumbens using a 1.5mm biopsy punch (Kai Medical Inc., Seki, Japan). Tissue samples were homogenised in 600 μ l of Trizol (Life Technologies, CA, USA) and 200 μ l of RNase-free H₂O (Life Technologies Corp., CA, USA), then mixed with 160 μ l of chloroform (Thermo Fisher Scientific Inc., Waltham, MA, USA) and phase separated by centrifuging for 15 minutes (12,000g) in pre-spun peqGOLD PhaseTrap A phase lock eppendorf tubes (PepLab Ltd., Erlangen, Germany).

2.2.3.2 RNA precipitation

The aqueous layer of each sample was decanted into an eppendorf tube (Sigma-Aldrich, Dorset, UK), then mixed with 0.5ml of isopropanol (Sigma-Aldrich, Dorset, UK), 50 μ l of sodium acetate (Sigma-Aldrich, Dorset, UK), and 4 μ l of glycoblue (Life Technologies Corp., CA, USA) and incubated at room temperature for 10 minutes. Samples were centrifuged (12,000g) at 4°C for 20 minutes until a RNA pellet formed, the supernatant was discarded and replaced with 1ml of 75% EtOH (Sigma-Aldrich, Dorset, UK) wash then centrifuged (7500g) for 5 minutes at 4°C. The wash was discarded, and pellets left to air dry for 30 minutes, then resuspended in 87.5 μ l of RNase-free H₂O (Sigma-Aldrich, Dorset, UK).

2.2.3.3. RNA cleanup

RNA was extracted using the RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Ltd., West Sussex, UK). To each 87.5 μ l solution; 10 μ l of buffer RDD and 2.5 μ l of DNase I stock solution were added and incubated at room temperature for 10 minutes. Then to this 100 μ l solution; 350 μ l of buffer RLT mixed with 3.5 μ l of β -mercaptoethanol and 250 μ l of 96-100% EtOH were mixed and immediately transferred to a spin column in a 2ml collection tube, then centrifuged (13,000g) for 15 seconds. Each spin column was transferred to a new collection tube to which 500 μ l of buffer RPE was added then centrifuged (13,000g) for 15 seconds. Each spin column was again transferred to a new collection tube to which 500 μ l of 80% EtOH was added then centrifuged (13,000g) for 15 seconds. Finally,

each spin column was transferred to a new collection tube and centrifuged (13,000g) for 5 minutes with the lid open. The spin columns were transferred to new 1.5µl eppendorf tubes to which 14µl of RNase-free H₂O was added and centrifuged (13,000g) for 2 minutes. Approximately 12µl of eluted RNA was retrieved.

2.2.3.4. RNA calculation and cDNA production

The concentration of RNA was determined using a NanoDrop 2000 micro-volume spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). A volume of RNA solution containing 1µg was added to the appropriate amount of RNase-free H₂O and 2µl of oligo(dT) primer (Life Technologies, CA, USA) to make a total volume of 15µl then incubated at 65°C for 5 minutes in a thermocycling PCR machine. Reactions were snap chilled on ice for 1 minute, after which 4µl of 5Xiscript select react mix (Life Technologies, CA, USA) and 1µl of reverse transcriptase (Life Technologies, CA, USA) were added to each. Finally, reactions were mixed and incubated at 42°C for 60 minutes then 85°C for 5 minutes in a thermocycling PCR machine, to make 20µl of cDNA.

2.2.3.5. qRT-PCR reaction

1µl of each cDNA sample (≤ 500 ng) was amplified by PCR in a 25µl reaction mixture; 12.5µl of SYBRGreen mastermix (Sigma-Aldrich, Dorset, UK), 0.6-µl of forward primer (primer sequences were designed using BLAST search with the NCBI tool Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/), presented in Table 2.1), 0.6-µl of reverse primer and 10.3µl of RNase-free H₂O, using an Mx4000 multiplex quantitative PCR sampler (Stratagene, La Jolla, CA, USA). Sample concentrations were calculated from serial dilution concentration curves, and each reaction was set up in triplicate, including GAPDH and 1µl RNase-free H₂O no template controls.

Primer	Forward	Reverse
GABA _A R α4- Exon 3	5' - CGTATTCTGGACAGTTTGCTGG ATGGT -3' (27)	5'- ACGGGCCCAAAGCTGGTGACAT -3' (22)
GAPDH	5'- TGCCCCCATGTTTGTGATG -3' (19)	5'- TGTGGTCATGAGCCCTTCC -3' (19)

Table 2.1. Primer sequences used for qRT-PCR analysis of constitutive and D1/D2-expressing neuron specific GABA_AR α4-subunit knockout mice, heterozygous and relative wildtype controls.

2.2.4. Production of Virus

2.2.4.1. AAV Production

Hek 293 Cells were cultured at 37 and 5% CO₂ in 90% Minimum Essential Medium [Invitrogen/Gibco, Cat. No. 21099-022] with GlutaMAX [Invitrogen/Gibco, Cat. No. 35050-038] and 10% heat inactivated horse serum, 1 mM Sodium Pyruvate (Invitrogen/Gibco, Cat. No. 11360-039), 0.1 mM non-essential amino acids (Invitrogen/Gibco Cat.No. 11140-35). Passaging was carried out using treatment of 0.5 mg/ml Trypsin; 0.2 mg/ml EDTA in PBS at intervals of 80-90% confluency.

Hek 293 cells were transfected at 70-80% confluency in 9cm plates. Plasmids were adjusted to 1mg/ml, then 10ug of pAAV-Cre or pAAV-GFP, pHelper and pRC plasmids were added to 1ml CaCl₂ and 1ml 2xHBS. DNA/CaCl₂/HBS mixture was applied to plates which were then incubated 6h at 37. 6h post transfection medium was aspirated and replaced with growth medium before incubation for a further 72h.

2.2.4.2 Harvesting AAVs

Cell/media suspension was decanted into a 50ml Falcon tube and cells were detached using a scraper into 2.5ml 1xDPBS then added to the same tube. Suspension was centrifuged at 20c for 5 minutes, then media was aspirated and

procedure to allow 4 plates per tube. Pellet was resuspended in 1068ul cell lysis buffer (3ml 5M NaCl, 5ml 1M Tris-HCl pH8, 80ml ddH₂O) then freeze-thawed 2x using dry-ice/37 water bath. Benzonase was added to the cell lysate at a final concentration of 50units/ml and incubated 30 ins at 37. Lysate was centrifuged at 3670g for 45 minutes at 4°C. Supernatant containing virus was collected and stored at -80°C.

2.2.5. Stereotaxic Viral Infusion

C57BL/6J mice anesthetised with isoflurane were stereotaxically infused with AAV-GFP or AAV-Cre, bilaterally into the NAc (coordinates AP1.34; L+/- 1.40; DV -4.20, (Paxinos and Franklin, 2001)). A steel infuser (33ga) connected via polyvinyl tubing to a (5µl) Hamilton Gastight syringe was used to infuse 1ul (0.5µl per side) of virus at a rate of 0.2µl/min for 5 minutes, then left to settle for an additional 5 minutes. Following surgery mice were singly housed and allowed to recover for 7 days.

2.2.6. Immunohistochemistry

Following experiments mice were euthanised by IP injection of Sodium Pentobarbital (10ml/kg). Mice brains were perfused via the aorta with 25ml (5ml/min) of phosphate buffer solution (PBS) followed by 75ml (5ml/min) of 4% paraformaldehyde (PF) (Sigma-Aldrich, St. Lois, MO, USA) in PBS. After perfusion, brains were removed and post-fixed overnight in 4% PF in PBS at 4°C, then transferred into 30% sucrose solution in PBS and left for 3 days at 4°C to cryoprotect. Coronal sections (30µm thick) were cut using a cryostat and collected in PBS-azide.

Free floating sections were washed 3 times in PBS for 10 minutes then incubated in blocking solution, 3% Normal Goat Serum (Vectorlabs) in PBS-T, for 1 hour with gentle agitation. Sections were then immediately incubated overnight in rabbit anti-GFP ployclonal primary antibody (1:10,000, Abcam ab6556) or rabbit anti-mCherry antibody (1:10,000, Abcam ab167453) diluted in blocking solution at 4°C. Sections were washed 3 times in PBS for 10 minutes before incubation in fluorescently tagged Alexafluor 488/568 anti-rabbit secondary antibodies (1:600, Thermofisher) diluted in blocking solution for 2 hours at room temperature.

2.2.7. Fluorescent in situ hybridisation (RNAScope)

2.2.7.1. Tissue Preparation

Mice were euthanised by intraperitoneal injection of sodium pentobarbital followed by cervical dislocation to minimise rupturing of blood vessels. Brains were extracted and flash-frozen by submergence in isopentane maintained at -50°C for 10 seconds then stored at -80°C prior to sectioning.

Brains were sectioned in an RNase free cryostat at -18°C. Brains were mounted on cryostat platforms using OCT mounting medium in -18°C chamber and left to equilibrate temperature for 1 hour prior to sectioning. Coronal sections of 10µm thickness were taken and mounted on SuperFrost Plus microscope slides (Thermofisher). Slides were stored at -80°C prior to in-situ-hybridisation.

Sections were submerged in 10% Buffered Formalin for 20 minutes at 4°C. Slides were washed in 1xPBS for 2 x 1 minute with gentle agitation then dehydrated in a series of ethanol solutions. Slides were submerged in 50% ethanol for 1 x 5 minutes, 70% ethanol 1 x 5 minutes and 100% ethanol for 2 x 5 minutes then incubated overnight in 100% ethanol at -20°C.

2.2.7.2. Procedure

In-situ-hybridisation was carried out using a manual RNAScope Fluorescent Multiplex Reagent Kit (Advanced Cell Diagnostics, Cat No. 320850) using instructions for fresh-frozen tissue and performing incubation steps using an ACD HybEZ™ Hybridization oven. We used RNAScope probes targeting: Mouse *Gabra4* (Cat No. 424261), Cre-recombinase (Cat No. 312281-C2), and either *Drd1* (Cat No. 406491-C3) or *Drd2* (Cat No. 406501-C3). We used the 'Amp4 Alt A' amplification reagent to label probes with fluorochromes as follows; *Gabra4* = Alexa-488, Cre-recombinase = Atto-647, and either *Drd1* or *Drd2* = Atto 550.

Images were captured using a QI click camera (Qimaging) attached to an Olympus Bx53 microscope (Olympus). Images of the NAc taken at 20x magnification were analyzed using ImageJ software (NIH).

2.2.8. Statistics

2.2.8.1. qRT-PCR

Data was analysed using the Mx4000 data analysis software (Stratagene, CA, USA), then exported to an Excel worksheet. Reaction triplicates were averaged, and then a mathematical model used to calculate the fold change of the target gene using the delta-delta CT of the target sample versus a control, expressed in comparison to the GAPDH reference gene (Pfaffl, 2001).

Expression of $\alpha 4$ subunit mRNA in the NAc or DStr of mice with intra-NAc expression of AAV-GFP or AAV-Cre were compared in an ANOVA using genotype as the between subjects variable and fold-change in expression relative to control as the dependant variable. In a subsequent, similar analysis $\alpha 4$ -WT and 'Floxed'- $\alpha 4$ groups were removed.

Expression of $\alpha 4$ subunit mRNA in the NAc and DStr of $\alpha 4$ -WT, 'Floxed'- $\alpha 4$ mice, and 'Floxed'- $\alpha 4$ mice with intra-NAc expression of AAV-GFP or AAV-Cre were compared in a repeated measures ANOVA using brain-region as the within subjects factor, genotype as the between subjects variable and fold-change in expression relative to control as the dependant variable. In a subsequent, similar analysis $\alpha 4$ -WT and 'Floxed'- $\alpha 4$ groups were removed.

2.2.8.2. Fluorescent in-situ-hybridisation (RNAscope)

We compared $\alpha 4$ mRNA expression in $\alpha 4$ -WT (n=3) and $\alpha 4$ -KO (n=3) mice. The amount of $\alpha 4$ mRNA probe signal was calculated as a percentage of the total image. This was averaged for each mouse and compared in a one-way ANOVA using genotype as the within subjects variable.

We compared $\alpha 4$, Cre, and D1 or D2 mRNA expression in $\alpha 4$ -D1-KO, $\alpha 4$ -D2-KO mice and their respective controls. Initially $\alpha 4$ -D1-WT and $\alpha 4$ -D2-WT were treated separately however we observed no differences and therefore grouped them as 'Floxed'- $\alpha 4$ mice in the presented analysis.

For images with multiple probes ($\alpha 4$, Cre, and D1 or D2) signal was measured in individual cells (see microscopy) and we calculated Pearson's correlation

coefficients to measure colocalization of signal from each probe. From these we calculated weighted mean correlations for each genotype using a Fisher-Z approach with Olkin and Pratt correction (as described by Olkin and Pratt, 1958; Alexander, 1990).

In order to compare the level of colocalization between $\alpha 4$ and D1 or D2 in the different genotypes we compared correlation coefficients for each pair of probes using the Fisher r-to-Z transformation method (Lenhard and Lenhard, 2014) in each genotype.

2.3. Results

2.3.1. Characterising constitutive $\alpha 4$ -KO mice

2.3.1.1. qRT-PCR for GABA_AR $\alpha 4$ -subunit in $\alpha 4$ -WT/Het/KO mice

To confirm deletion of $\alpha 4$ mRNA transcript we performed a quantitative RT-PCR experiment using primers directed to exon 3. The qRT-PCR analysis revealed GABA_AR $\alpha 4$ -subunit mRNA levels to be reduced in NAc of $\alpha 4$ -Het mice and completely absent in $\alpha 4$ -KO mice when compared to $\alpha 4$ -WT control mice, confirming the knockout of $\alpha 4$ (Table 2.2, Figure 2.5; significant main effect of genotype, $F_{(2,9)} = 666.42$, $p < 0.001$). We therefore confirm complete deletion of exon 3 in $\alpha 4$ -KOs and ~50% expression in $\alpha 4$ -Hets.

GABA_AR $\alpha 4$ -subunit mRNA levels were similarly reduced in the DStr of $\alpha 4$ -Het mice and again completely absent in $\alpha 4$ -KO mice when compared to $\alpha 4$ -WT control mice, further confirming the knockout of $\alpha 4$ throughout the striatum (Table 2.2, Figure 2.5; significant main effect of genotype, $F_{(2,9)} = 44.406$, $p < 0.001$). We therefore confirm complete deletion of $\alpha 4$ in $\alpha 4$ -KOs and ~50% expression in $\alpha 4$ -Hets.

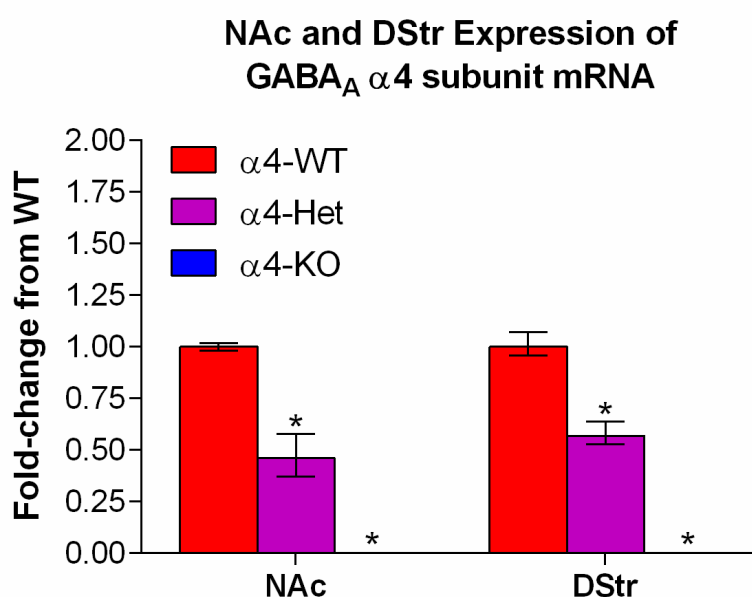


Figure 2.5. Fold change from WT controls of GABA_AR α 4 subunit mRNA expression in the NAc and DStr of α 4-WT (n=4), α 4-Het (n=4) and α 4-KO (n=4) mice. GABA_AR α 4-subunit expression in the NAc and Dorsal Striatum was absent in α 4-KO mice and reduced to ~50% in α 4-Het mice ($p<0.001$ *). Error bars represent SEM.

Region	Genotype	Fold change	% Change from WT	Sig
NAc	α 4-WT	1 \pm 0.02/0.02	0%	$p<0.001$
	α 4-Het	0.93 \pm 0.02/0.05	-54%	
	α 4-KO	0.9 \pm 0.09/0.07	-100%	
DStr	α 4-WT	1 \pm 0.12/0.09	0%	$p<0.001$
	α 4-Het	0.98 \pm 0.07/0.04	-47%	
	α 4-KO	0.9 \pm 0.09/0.07	-10%	

Table 2.2 NAc and DStr mRNA expression levels of GABA_AR α 4 subunit in α 4-WT (n=4), α 4-Het (n=4) and α 4-KO (n=4) mice were compared in triplicate against α 4-WT mice to give a measure of fold change. Fold change from WTs was tested statistically using Least-Square-Difference post hoc comparisons.

2.3.1.2. Fluorescent in situ hybridisation (RNAScope) for GABA_AR α 4-subunit in α 4-WT/KO mice

In order to visualise expression of the GABA_A α 4 subunit within the striatum we performed a fluorescence in situ hybridisation on brain sections taken from wildtype (α 4-WT, n=3) constitutive α 4 knockout (α 4-KO, n=3) mice.

Surprisingly we detected α 4 mRNA signal in both the α 4-Wt *and* α 4-KO mice (Figure 2.6). We therefore quantified the amount of signal in images of the NAc and compared this between α 4-WT and α 4-KO mice. The α 4 mRNA signal was significantly reduced in α 4-KO mice relative to α 4-WT (Figure 2.6, significant main effect of genotype, $F_{(1,5)} = 19.21$, $P < 0.05$). Although we detected some background signal it is therefore possible to distinguish α 4-WT and α 4-KO mice via in situ hybridisation.

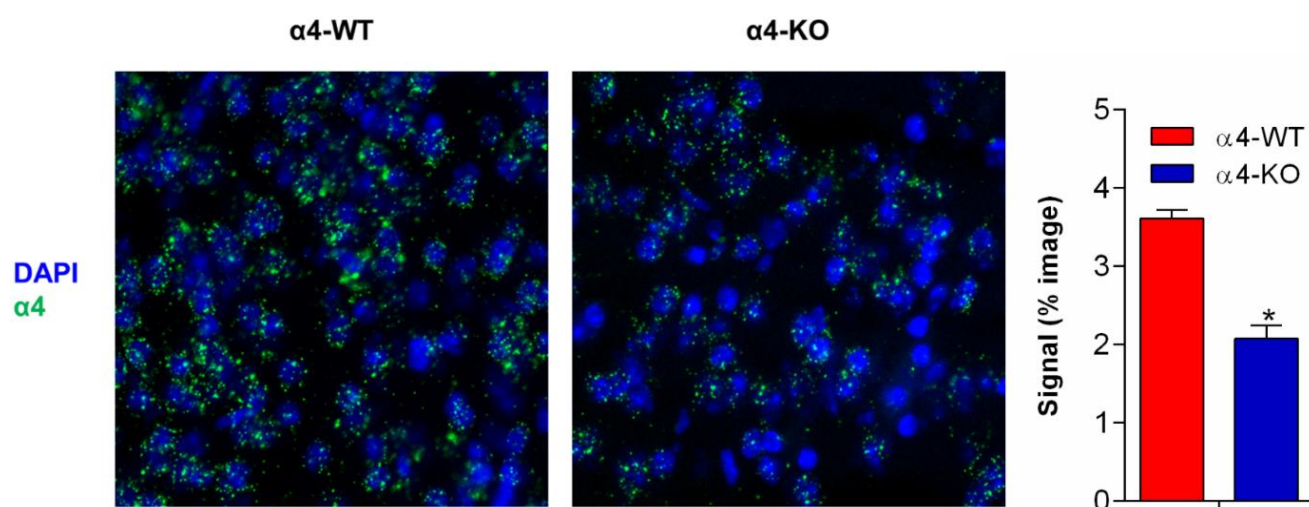


Figure 2.6. In-situ-hybridisation for GABA_A α 4 subunit mRNA in the NAc of α 4-Wt and α 4-KO mice. Neurons are highlighted by DAPI staining (blue) and the probe targets α 4 mRNA (green). Signal is significantly reduced in α 4-KO mice compared with α 4-WT ($p < 0.05$). Error bars represent SEM.

2.3.2. Characterising D1/D2 specific $\alpha 4$ KOs

2.3.2.1. Fluorescent in situ hybridisation (RNAScope) Cre and D1/D2

To confirm that Cre was correctly expressed only in D1 or D2 neurons of $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice respectively we performed a fluorescent in situ hybridisation using probes for $\alpha 4$, Cre-recombinase, and either D1R or D2R on NAc brain sections from 'Floxed'- $\alpha 4$, $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice (Figure 2.7).

In 'Floxed'- $\alpha 4$ mice we found no expression of Cre as expected. We detected significantly greater expression of Cre in $\alpha 4$ -D2-KO mice compared to $\alpha 4$ -D1-KO mice (Figure 2.7).

In $\alpha 4$ -D1-KO mice ($n=3$) Cre was positively correlated with D1 ($r=0.237$, $t_{(307)}=4.271$, $p<0.001$) but not with D2 ($r=-0.0023$, $t_{(393)}=-0.045$, $p = 0.096$, NS) and not with Cre ($r=-0.0$, $t_{(393)}=-0.045$, $p = 0.096$, NS).

Conversely, in $\alpha 4$ -D2-KO mice ($n=3$) Cre was negatively correlated with D1 ($r=-0.279$, $t_{(413)}=-5.92$, $p<0.001$, NS) but was positively correlated with D2 ($r=0.74$, $t_{(411)}=22.54$, $p<0.001$).

Genotype	Correlation	Pearson's r	Sig
'Floxed'- $\alpha 4$	$\alpha 4$ & D1	+0.304	$p<0.001$
	$\alpha 4$ & D2	+0.305	$p<0.001$
$\alpha 4$ -D1-KO	Cre & D1	+0.237	$p<0.001$
	Cre & D2	-0.002	NS
	Cre & $\alpha 4$	+0.079	NS
	$\alpha 4$ & D1	+0.213	$p<0.001$
	$\alpha 4$ & D2	+0.369	$p<0.001$
$\alpha 4$ -D2-KO	Cre & D1	-0.279	$p<0.001$
	Cre & D2	+0.74	$p<0.001$
	Cre & $\alpha 4$	+0.08	NS
	$\alpha 4$ & D1	+0.484	$p<0.001$
	$\alpha 4$ & D2	+0.182	$p<0.001$

Table 2.3. Table of correlations between signal from probes targetting GABA_A $\alpha 4$ subunit, Cre-recombinase and either D1R or D2R in the NAc of each genotype.

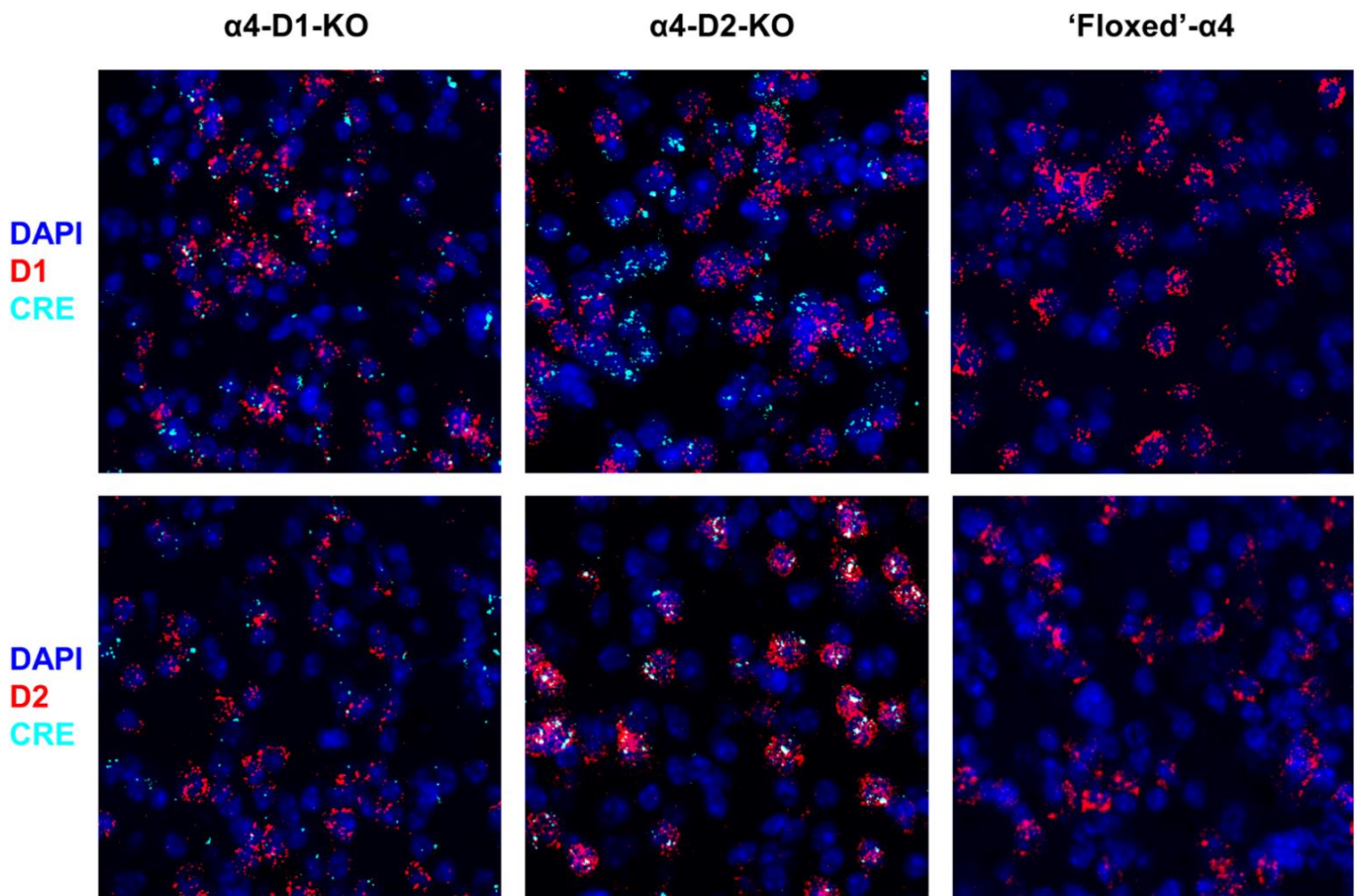


Figure 2.7. In-situ-hybridisation for Cre-recombinase and D1/D2 mRNA in the NAc of $\alpha 4$ -D1-Ko, $\alpha 4$ -D2-KO and 'Floxed'- $\alpha 4$ mice. Neurons are highlighted by DAPI staining (blue) and probes target Cre-recombinase (cyan) and D1 (red, upper panels) or D2 (red, lower panels) mRNA by probes (green). In $\alpha 4$ -D1-KO animals Cre positively correlates with D1 but not D2 ($p < 0.001$). In $\alpha 4$ -D2-KO animals Cre positively correlates with D2 ($p < 0.001$) and negatively correlates with D1 ($p < 0.001$).

2.3.2.2. Fluorescent in situ hybridisation (RNAscope) for $\alpha 4$, Cre and D1/D2

We did not observe a significant difference in $\alpha 4$ expression between 'Floxed' $\alpha 4$, $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO (non-significant main effect of genotype, $F_{(2,20)} = 3.25$, $p = 0.13$).

In 'Floxed'- $\alpha 4$ mice $\alpha 4$ was positively correlated with D1 ($r=0.304$, $t_{(282)}=5.368$, $p<0.001$) and with D2 ($r=0.305$, $t_{(464)}=6.88$, $p<0.001$). Comparison of Pearson's coefficients found that $\alpha 4$ colocalised to a similar degree with D1 and D2 (Table 2.3, $z=0$, $p=0.5$).

In $\alpha 4$ -D1-KO mice $\alpha 4$ was positively correlated with D1 ($r=0.213$, $t_{(309)}=$, $p<0.001$) and with D2 ($r=0.369$, $t_{(393)}=7.85$, $p<0.001$) but not with Cre ($r=0.0793$, $t_{(333)}=1.394$, $p=0.16$). Comparison of Pearson's coefficients found that $\alpha 4$ was more strongly colocalised with D2 than D1 (Table 2.3, $z=-2.22$, $p<0.05$).

In $\alpha 4$ -D2-KO mice $\alpha 4$ was positively correlated with D1 ($r=0.484$, $t_{(413)}=11.25$, $p<0.001$) and with D2 ($r=0.182$, $t_{(411)}=3.76$, $p<0.001$) but not with Cre ($r=0.08$, $t_{(413)}=1.65$, $p=0.1001$, NS). Comparison of Pearson's coefficients found that $\alpha 4$ was more strongly colocalised with D1 than with D2 (Table 2.3, $z=5$, $p<0.001$).

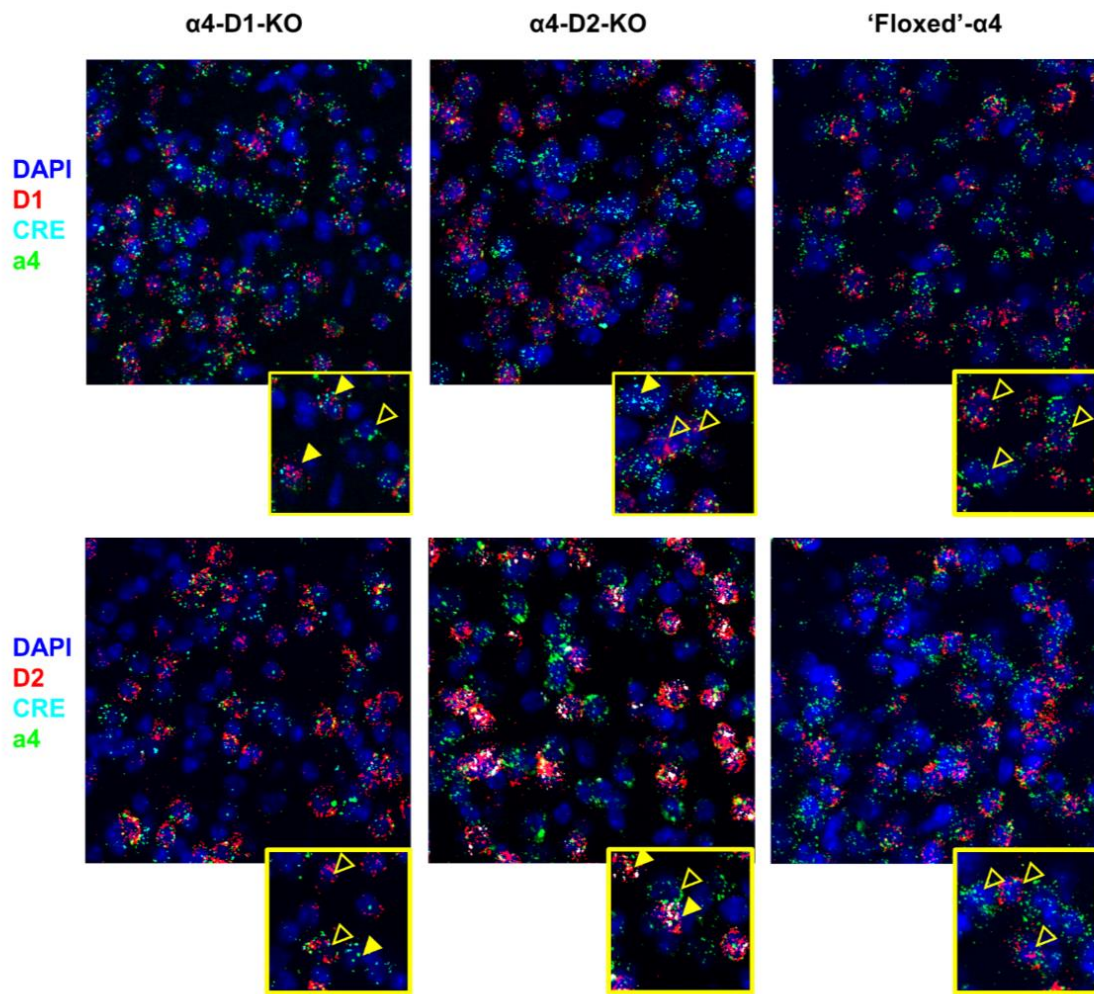


Figure 2.8. In-situ-hybridisation for GABA_A $\alpha 4$ subunit, Cre-recombinase and D1/D2 mRNA in the NAc of $\alpha 4$ -D1-KO, $\alpha 4$ -D2-KO and 'Floxed'- $\alpha 4$ mice. Neurons are highlighted by DAPI staining (blue) and probes target $\alpha 4$ subunit (green) Cre-recombinase (cyan) and D1 (red, upper panels) or D2 (red, lower panels) mRNA by probes (green). Boxes highlight Cre expressing (solid arrows) and non-cre-expressing (transparent arrows) neurons. GABA_A $\alpha 4$ subunit correlates equally with D1 and D2 in 'Floxed'- $\alpha 4$ mice. GABA_A $\alpha 4$ subunit correlates less strongly with D1 in $\alpha 4$ -D1-KO mice ($p < 0.001$) and less strongly with D2 in $\alpha 4$ -D2-KO mice ($p < 0.001$).

2.3.3 Characterising Cre virus in vivo

2.3.3.1. Confirming AAV expression by Immunohistochemistry

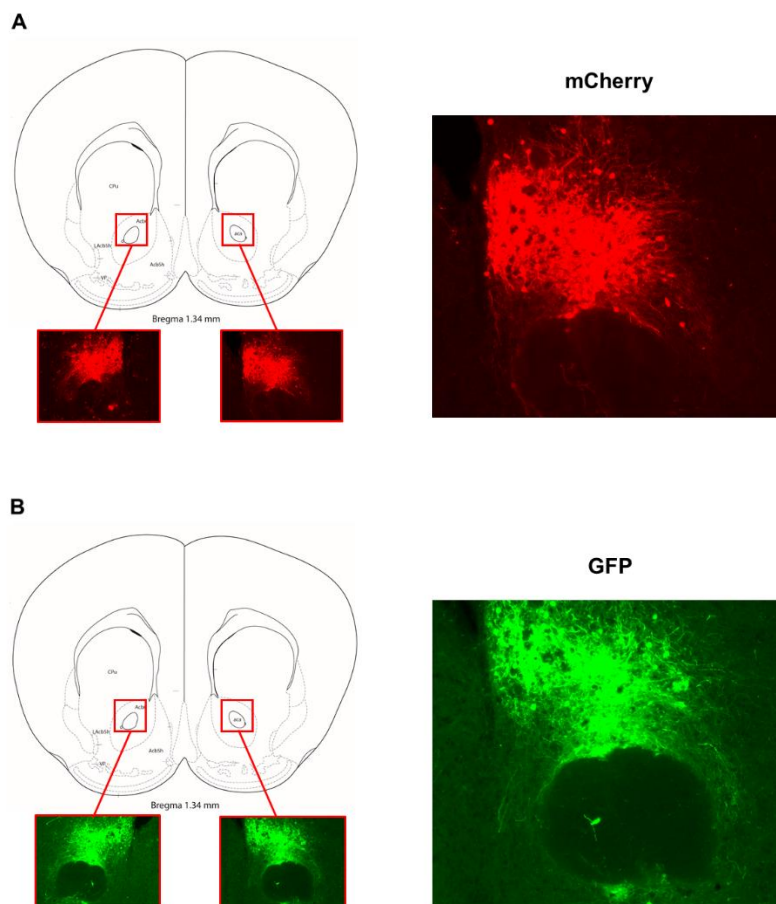


Figure 2.9. (A) Expression of AAV-Cre virus in the NAc of 'Floxed'- α 4 mice visualised by immunohistochemistry using antibody for mCherry marker. (A) Expression of AAV-Cre virus in the NAc of 'Floxed'- α 4 mice visualised by immunohistochemistry using antibody for GFP marker.

2.3.3.2. Confirming AAV-Cre knockdown of α 4 by qRT-PCR

To test whether wildtype and 'floxed'- α 4 mice have similar levels of α 4 subunit expression and whether the AAV-Cre virus was able to reduce α 4 subunit mRNA expression in the NAc of 'Floxed'- α 4 mice we used a qRT-PCR experiment to compare α 4-WT (n=4), 'Floxed'- α 4 (n=4) mice, and 'Floxed'- α 4 mice with intra-

NAc expression of AAV-GFP (n=4) or AAV-Cre (n=4) to give a measure of fold change compared to $\alpha 4$ -WT mice.

In the NAc $\alpha 4$ expression was reduced in 'floxed'- $\alpha 4$ + AAV-Cre mice but not in untreated 'Floxed'- $\alpha 4$ or 'Floxed'- $\alpha 4$ + AAV-GFP mice (Figure 2.10, Table 2.4, significant main effect of genotype, $F_{(3,12)} = 6.2$, $p < 0.05$, post hoc WT vs Floxed + Cre, $p < 0.05$). In a control region, the DStr, $\alpha 4$ expression was similar to wildtypes in untreated 'Floxed'- $\alpha 4$, 'Floxed'- $\alpha 4$ + AAV-GFP or 'Floxed'- $\alpha 4$ + AAV-Cre mice (Figure 2.10, Table 2.4, non-significant main effect of genotype, $F_{(3,12)} = 0.48$, $p = 0.985$, NS).

Further, to determine that $\alpha 4$ subunit expression was reduced by accurate injection of AAV-Cre in the NAc of Floxed- $\alpha 4$ mice we compared mRNA expression in both the NAc and control region, the DStr, of these mice in a repeated measures analysis. We found that $\alpha 4$ expression was reduced only in the NAc but not DStr of 'Floxed'- $\alpha 4$ + AAV-Cre mice (Figure 2.10, significant region by genotype interaction $F_{(1,12)} = 19.07$, $p < 0.001$).

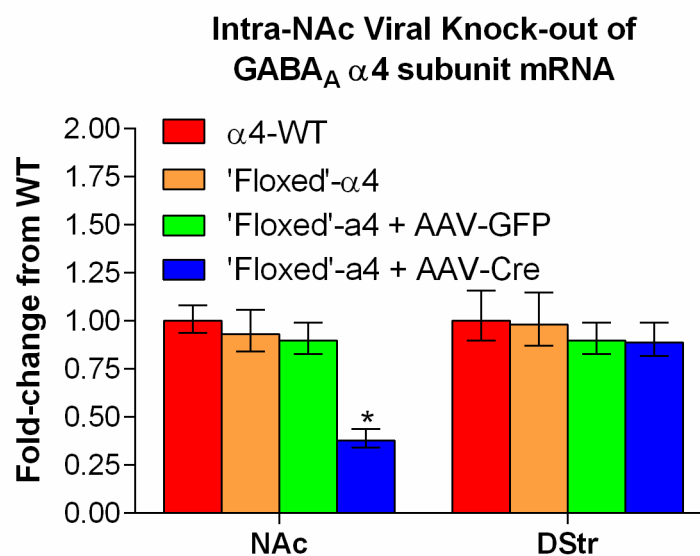


Figure 2.10. Fold change from WT controls of GABA_A $\alpha 4$ subunit mRNA expression in the NAc and DStr of $\alpha 4$ -WT (n=4) mice, 'Floxed'- $\alpha 4$ (n=4) mice, and 'Floxed'- $\alpha 4$ mice with intra-NAc expression of AAV-GFP (n=4) or AAV-Cre (n=4). 'Floxed'- $\alpha 4$ mice with intra-NAc expression of AAV-Cre (n=4) show a decrease in $\alpha 4$ expression in the NAc ($p < 0.05^*$) but not DStr. Error bars represent SEM.

Region	Genotype	Fold change	% Change from WT	Sig
NAc	$\alpha 4$ -WT	$1 \pm 0.08/0.06$	0%	
	'Floxed'- $\alpha 4$	$0.93 \pm 0.13/0.09$	-7%	NS
	AAV-GFP	$0.9 \pm 0.09/0.07$	-10%	NS
	AAV-Cre	$0.38 \pm 0.06/0.04$	62%	$P = 0.023^*$
DStr	$\alpha 4$ -WT	$1 \pm 0.16/0.1$	0%	
	'Floxed'- $\alpha 4$	$0.98 \pm 0.17/0.11$	-2%	NS
	AAV-GFP	$0.9 \pm 0.09/0.07$	-10%	NS
	AAV-Cre	$0.89 \pm 0.1/0.07$	-11%	NS

Table 2.4. NAc and DStr mRNA expression levels of GABA_AR $\alpha 4$ subunit in $\alpha 4$ -WT, 'Floxed'- $\alpha 4$, and 'Floxed'- $\alpha 4$ mice with intra-NAc expression of AAV-GFP (n=4) or AAV-Cre (n=4) were compared in triplicate against $\alpha 4$ -WT mice with to give a measure of fold change. Fold change from WTs was tested statistically using Least-Square-Difference post hoc comparisons.

'Floxed'- $\alpha 4$ + AAV-GFP mice provide the best physiological control for 'Floxed'- $\alpha 4$ + AAV-GFP mice and, as outlined above, did not differ significantly from wildtype or 'Floxed'- $\alpha 4$ mice. We therefore performed a subsequent analysis in which we compared $\alpha 4$ subunit fold change in the NAc or DStr of 'Floxed'- $\alpha 4$ + AAV-Cre compared with 'Floxed'- $\alpha 4$ + AAV-GFP controls. In the NAc $\alpha 4$ expression was reduced in 'Floxed'- $\alpha 4$ + AAV-Cre mice (Figure 2.11, Table 2.5, significant main effect of genotype, $F_{(1,6)} = 20.16$, $p < 0.005$). In the DStr $\alpha 4$ expression was similar in 'Floxed'- $\alpha 4$ + AAV-GFP and 'Floxed'- $\alpha 4$ + AAV-Cre mice (Figure 2.11, Table 2.5, non-significant main effect of genotype, $F_{(1,6)} = 20.16$, $p < 0.005$). We found that $\alpha 4$ expression was reduced only in the NAc but not DStr of 'Floxed'- $\alpha 4$ + AAV-Cre mice (Figure 2.11, significant region by genotype interaction $F_{(1,12)} = 33.46$, $p < 0.001$).

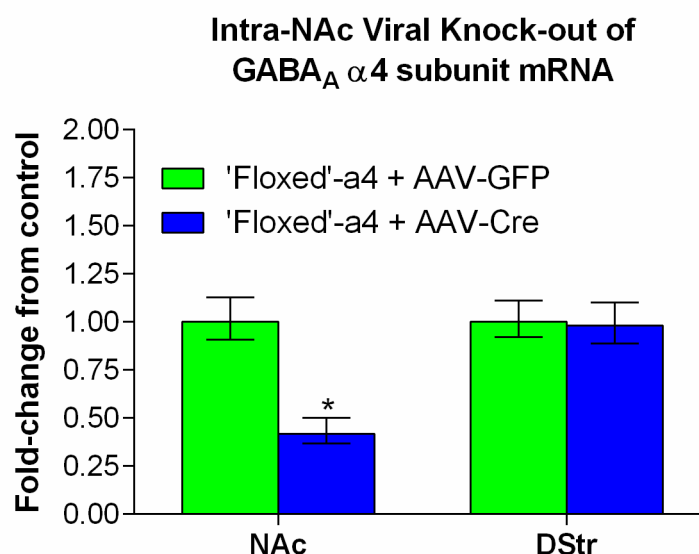


Figure 2.11. Fold change from 'Floxed'- α 4 + AAV-GFP controls (n=4) of GABA_AR α 4 subunit mRNA expression in the NAc and DStr of 'Floxed'- α 4 + AAV-Cre mice (n=4). 'Floxed'- α 4 mice with intra-NAc expression of AAV-Cre (n=4) show a decrease in α 4 expression in the NAc ($p < 0.05^*$) but not DStr. Error bars represent SEM.

Region	Genotype	Fold change	% Change from Cntrl	Sig
NAc	AAV-GFP	1 \pm 0.13/0.09	0%	$P = 0.004$
	AAV-Cre	0.42 \pm 0.08/0.05	-58%	
DStr	AAV-GFP	1 \pm 0.11/0.08	0%	NS
	AAV-Cre	0.98 \pm 0.12/0.09	-2%	

Table 2.5. NAc and DStr mRNA expression levels of GABA_AR α 4 subunit in 'Floxed'- α 4 mice with intra-NAc expression of AAV-GFP (n=4) or AAV-Cre (n=4) were compared in triplicate against 'Floxed'- α 4 + AAV-GFP (n=4) to give a measure of fold change. Fold change from WTs was tested statistically using Least-Square-Difference post hoc comparisons.

2.4. Discussion

2.4.1. Constitutive GABA_A α 4 subunit Knockout Mice

Our genotyping procedure and qRT-PCR analysis of NAc and DS tissue samples confirmed the absence of GABA_A α 4-subunit DNA and mRNA expression in α 4-KO mice, and a reduction of approximately 50% in α 4-Het mice when compared to α 4-WT controls. These data confirm cre/loxp cleavage of the *gabra4* gene.

To visualise the expression pattern of α 4, to enable investigation of colocalisation with other neural markers in subsequent analysis, we used a recently developed fluorescent in-situ-hybridisation method (RNAscope). This method has been useful in other studies of low expressing genes as it amplifies mRNA signal for clear visualisation (Wang *et al.*, 2012). Unexpectedly, we appeared to detect signal from the probe targeting the *gabra4* gene in α 4-KO animals where it should be absent.

Both genotyping and qRT-PCR analysis confirmed absence of exon-3 of the *gabra4* gene in addition to previous studies which have demonstrated a lack of GABA_A α 4 subunit protein in α 4-KO mice from the same colony via both Western blot and immunohistochemistry (see figure 2.12. below), as well as electrophysiological differences (Macpherson, 2013; Maguire *et al.*, 2014).

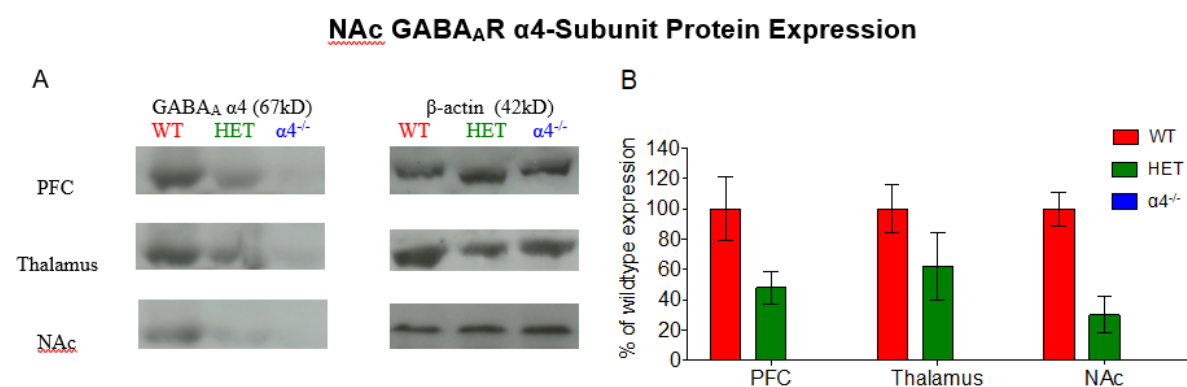


Figure 2.12. Western Blot analysis of PFC, thalamus and NAc tissue from GABA_A α 4-WT (WT, n=3), α 4-Het (HET, n=3) α 4-KO (α 4^{-/-}, n=3) mouse tissue (Macpherson, 20313). (A) Representative images of western blot results for GABA_A α 4 and β -actin. Blots probed for β -actin show equal loading of samples. (B) Percentage change from α 4-WTs of the protein GABA_A α 4 in the PFC,

thalamus and NAc. The expression of the GABA_A $\alpha 4$ protein was lacking in all tested brain regions of the $\alpha 4$ -KO mice and significantly reduced in $\alpha 4$ -Het mice.

We therefore suggest that $\alpha 4$ signal observed in $\alpha 4$ -KO mice results from either non-specific binding of the probe or that following deletion of exon-3 in $\alpha 4$ -KO mice there remains a truncated mRNA transcript which does not lead to expression of functional $\alpha 4$ protein but is able to bind the RNAscope probe. The primers used in genotyping and qRT-PCR analysis are targeted within the deleted exon-3 whereas the RNAscope probe targets a 1316 base-pair region (449 to 1765) surrounding exon-3 but encompassing upstream and downstream regions including exons 1-8. If a truncated mRNA is present it could therefore have a large amount of sequence homology with the $\alpha 4$ probe. Despite this problematic 'background' signal we observed higher levels of $\alpha 4$ mRNA specific signal in $\alpha 4$ -WT mice and are therefore able to distinguish $\alpha 4$ -WT and $\alpha 4$ -KO neurons using this method.

We attempted to investigate the possibility of a truncated transcript using qRT-PCR with two pairs of primers directed either upstream or downstream of exon 3. Unfortunately, these primers failed to replicate cDNA in either $\alpha 4$ -KO or $\alpha 4$ -WT mice. Further work to develop effective qRT-PCR primers for exons 1/2 or 4-8 may confirm or disprove the presence of a truncated $\alpha 4$ mRNA in $\alpha 4$ -KO mice. Alternatively, a new *gabra4* RNAscope probe could be developed using, if possible, a smaller sequence or one further upstream of exon-3.

2.4.2. D1- and D2- specific GABA_AR $\alpha 4$ subunit Knockout Mice

To confirm the D1- and D2-specific knockout of $\alpha 4$ in our conditional knockout $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice we performed in-situ-hybridisation using probes targeting Cre-recombinase, *gabra4*, and either *Drd* or *Drd2*. No Cre was detected in 'Floxed'- $\alpha 4$ mice (either $\alpha 4$ -D1-WT or $\alpha 4$ -D2-WT) which serve as controls for the conditional $\alpha 4$ knockout mice. In $\alpha 4$ -D1-KO mice Cre was colocalized with D1 but not D2 whereas in $\alpha 4$ -D2-KO mice Cre colocalized with D2 and was negatively correlated with D1. We have therefore confirmed that Cre is correctly expressed in the expected neural populations according to the original driver lines. Overall, Cre expression was higher in the NAc of $\alpha 4$ -D2-KO mice than $\alpha 4$ -D1-KO mice. This is also expected as Cre expression was higher in

the D1- than D2- BAC-Cre founder line used to breed our conditional knockouts (Gong *et al.*, 2007).

The difference in Cre expression may affect our interpretation of behavioural differences in these mice as the $\alpha 4$ subunit will be deleted in a smaller population of neurons in D1 than D2 mice which may result in a less severe phenotype. Further, in the D2 BAC founder line Cre is more broadly expressed throughout the Dorsal and Ventral striatum whilst in the D1 line it is more restricted to the NAc. This adds anatomical specificity to the D1 manipulation but makes comparisons of behaviour between D1- and D2- knockouts less readily attributable to D1/D2 neural dissociation and potentially partially attributable to anatomical differences. However, our previous studies and this thesis focus on drug potentiated behaviours and conditioning which are thought to be primarily mediated by the NAc where Cre is expressed in both lines. We should exercise some caution when interpreting behaviours mediated by the Dorsal Striatum, such as baseline locomotor activity.

In 'floxed- $\alpha 4$ ', $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice, $\alpha 4$ was colocalized with both D1 and D2. Our analysis revealed that in 'floxed'- $\alpha 4$ mice this correlation was equal in D1 and D2 neurons. In contrast, we found that in $\alpha 4$ -D1-KO mice $\alpha 4$ was more strongly correlated with D2 and less with D1, whereas in $\alpha 4$ -D2-KO mice the reverse was true; $\alpha 4$ was more strongly correlated with D1 and less with D2. We therefore conclude that the presence $\alpha 4$ probe signal in both populations is due to the background signal (discussed above) and populations of D1 and D2 neurons not expressing Cre in $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice respectively due to position effect variegation. Our results suggest a reduction of the $\alpha 4$ mRNA specific signal in D1 or D2 neurons of $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice respectively.

The overall level of $\alpha 4$ mRNA signal was not significantly different between genotypes. We may have expected a reduction in $\alpha 4$ conditional knockouts relative to 'floxed'- $\alpha 4$ controls based on previous qRT-PCR data (Macpherson, 2013). The high level of signal amplification in the RNAscope method makes quantification difficult and may mask such smaller differences in expression (Wang *et al.*, 2012).

2.4.3. AAV-Cre virus mediated NAc specific Knockout of the GABA_AR α 4 subunit

Finally, we used an AAV viral vector to deliver Cre to the NAc of 'Floxed'- α 4 mice and demonstrated its ability to reduce α 4 subunit mRNA. Expression of the AAV-Cre virus in the NAc was confirmed by immunohistochemistry for the mCherry marker. We were able to visualise mCherry expression within the targeted region of the NAc core to confirm localisation of the virus. Further qRT-PCR analysis demonstrated substantial knockdown (~60%) of GABA_AR α 4 subunit mRNA in the NAc but not in a nearby control region (Dorsal Striatum) when compared with the control AAV-GFP virus or untreated mice. Importantly, we also demonstrated similar α 4 expression in 'Floxed'- α 4 animals as in α 4-WT mice which verifies them as equally ecologically representative controls in experiments using manipulations of α 4.

One of the primary advantages of using viral vectors is to eliminate the compensatory mechanism observed in several GABA_AR subunit knockout mice (Brickley *et al.*, 2001; Sur *et al.*, 2001; Vicini *et al.*, 2001). However, under certain conditions, such as cocaine or alcohol treatment, expression of GABA_AR subunits has been found to be rapidly modulated (Liang *et al.*, 2007; Heiman *et al.*, 2008). It would therefore be of interest to perform further qRT-PCR experiments analysing expression of various other GABA_AR subunits in NAc tissue from 'Floxed'- α 4 mice treated with AAV-Cre. If other subunits are upregulated their compensatory effects could mask behavioural effects of the localised α 4 knockout.

The presented data, in addition to previous studies, demonstrate that the GABA_AR α 4 subunit is functionally deleted in α 4-KO mice. Further, in D1- and D2- specific conditional knockout mice Cre/loxp deletion has reduced α 4 expression in D1 or D2 expressing neural populations respectively. We have also produced an AAV-Cre virus which can be used to locally knockdown α 4 expression when surgically injected into a region of interest, the NAc.

Chapter 3

The role of $\alpha 4$ -containing GABA_A receptors in mediating binge-like alcohol drinking (Drinking in the Dark)

3.1. Introduction

3.1.1 Pre-clinical models of alcohol drinking

Globally harmful use of alcohol has been estimated to cause 5.9% of annual deaths and account for 5.1% of the global health burden (WHO, 2014). In particular 'binge drinking', a pattern in which blood alcohol levels are raised to 0.08 grams alcohol per decilitre blood (0.8mg/ml), is associated with a large proportion of alcohol related deaths, diseases and social harms (NIAAA, 2004).

Pre-clinical animal models, typically rodent, allow the use of invasive pharmacological and genetic techniques in combination with behavioural tests to identify neuronal systems underlying the reinforcing effects of alcohol. A number of techniques have been developed in which ethanol consumption and preference can be measured (reviewed, Crabbe et al. 2011). Of these two of the most commonly used procedures are 'Two-Bottle Choice' and 'Drinking in the Dark' (DID).

In 'Two-Bottle Choice' procedures an ethanol solution is presented alongside drinking water and can be provided continuously or under chronic, intermittent access conditions (24h access on alternating days) (Richter & Campbell 1940, Wise 1973). Drinking in the Dark (DID) is a limited access procedure in which the water supply of a rat or mouse is exchanged for an ethanol solution (10 to 20% ABV) during a two hour period in the dark phase of the light cycle and consumption is recorded (Ryabinin et al. 2003, Rhodes et al. 2005). Ethanol consumption in these studies was high enough to reliably predicted blood alcohol concentrations (BACs) >1mg/ml in C57BL/6J mice. An obvious problem with DID is the lack of choice available however a 2h period of voluntary water deprivation is not nearly sufficient to challenge mice physiologically (Toth & Gardiner 2000, Crabbe et al. 2011). Under conditions of intermittent access (Wise, 1973) and to a greater extent limited access (Rhodes *et al.*, 2005) animals show an increased

preference for alcohol and achieve higher BACs which makes them the best models of 'binge drinking' behaviour.

These procedures are informative about alcohol consumption but not the motivation for ethanol consumption or its reinforcing properties. DID ethanol consumption and BACs are not changed by food deprivation or when peripheral leptin/ghrelin administration was used to decrease/increase feeding in C57BL/6J mice (Lyons *et al.*, 2008) implying that increased consumption is not due to calorie seeking. Even so, these models only examine the consummatory phase of behaviour rather than the motivational or appetitive. Procedures have therefore been developed to train animals to respond for ethanol reinforcement in operant conditioning tasks where rodents are trained to press levers to receive small volumes of ethanol solution as reinforcers (Samson, 1986). Due to the aversive taste of alcohol this typically requires a 'sucrose fading' technique whereby food deprived animals are trained to respond for solutions in which sucrose content is incrementally reduced and ethanol content is increased over an 'acquisition phase' (Elmer, Meisch and George, 1986; Samson, 1986; Samson, Pfeffer and Tolliver, 1988).

Although operant self-administration experiments provide insight into motivational behaviour sucrose fading introduces the confound of an additional rewarding stimuli and typically rodents do not reach such high BACs as in DID procedures (Elmer, Meisch and George, 1986; Rhodes *et al.*, 2005; Crabbe, Harris and Koob, 2011; Doherty and Gonzales, 2015). Additionally studies that simultaneously recorded operant responses and volume/rate of subsequent ethanol consumption found that higher initial operant responses did not predict larger or faster drinking bouts therefore dissociating appetitive and consummatory aspects of drinking behaviour (Samson *et al.*, 1998). This suggests that aside from appetitive mechanisms consummatory behaviour is an important component of binge-like drinking itself.

3.1.2. GABA in ethanol consumption and reinforcement

Alcohol can be described as lacking molecular specificity in comparison to other drugs of abuse which have specific molecular targets (e.g. heroin at opioid receptors or cocaine at dopamine transporters). Rather alcohol has low affinity

interactions with many different targets including neurotransmitters, receptors, enzymes and other molecules (Koob, 2014; Ron and Barak, 2016).

Of the neurotransmitters and receptors affected by alcohol GABAergic systems appear to be a prominent target of alcohol (Davies, 2003). GABAergic components of ethanol related behaviour are varied as receptors mediate different effects depending on their class and sub-unit composition (George F Koob, 2004). Pharmacological manipulations of different GABA receptor classes have revealed different and often opposing roles of ionotropic (GABA_A) and metabotropic (GABA_B) receptors in alcohol consumption and reinforcement (reviewed below).

There is some convergence of pre-clinical evidence that agonism of GABA_B receptors reduces alcohol consumption, motivation and withdrawal. Intraperitoneal (IP) injection of the GABA_B receptor agonist baclofen dose dependently reduced ethanol consumption in rats during continuous access in the 'Two-bottle choice' procedure (Colombo *et al.*, 2000) and operant self-administration (Anstrom *et al.*, 2003). IP baclofen and SKF 97541 (another GABA_B agonist) also reduced operant self-administration of ethanol but also saccharin solution in C57BL/6J mice (Besheer, Lepoutre and Hodge, 2004) although this may have been due to potentiation of alcohol's sedative effects.

In contrast opposite effects have been found using modulators of GABA_A receptor activity. The GABA_A antagonist RO15-4513 reduced ethanol self-administration in alcohol preferring Wistar rats (Rassnick *et al.*, 1993). Voluntary ethanol consumption in rats (measured by Two-Bottle Choice) can be decreased by IP injection of the GABA_A *antagonist* picrotoxin but is increased by the GABA_A *agonist* THIP (Boyle *et al.*, 1993) indicating bidirectional control of ethanol consumption via GABA_A receptors.

The effects of GABAergic compounds can be localised to specific brain regions. GABA receptors are expressed in many brain areas, including the mesolimbic dopamine system, associated with drug and ethanol reinforcement (reviewed; Koob 2004). Injections of potent GABA_A antagonist SR 95531 into the central amygdala (CA) or Nucleus accumbens (NAc) were sufficient to suppress responding in ethanol self-administration (Hyytiä and Koob, 1995). Similarly,

infusion of bicuculline or muscimol into the NAc suppressed ethanol self-administration. The NAc is a major region involved in the reinforcing properties of drugs of abuse including alcohol (Koob and Volkow, 2010). Rats self-administer ethanol directly to the NAc and alcohol preferring rats do so to a greater extent (Engleman *et al.*, 2009) indicating that alcohol acts directly in this region to mediate its reinforcing properties.

GABA may also mediate changes in striatal medium spiny neurons (MSNs) that are involved in the acquisition of alcohol dependence. Repeated ethanol exposure in a DID paradigm resulted in an increase in both consumption and drinking rate associated with a reduced frequency of GABA_A receptor mediated inhibitory postsynaptic currents in striatal MSNs (Wilcox *et al.*, 2014). These results indicate that synaptic GABA_A transmission in MSNs is decreased following chronic or binge-like alcohol consumption.

3.1.3. $\alpha 4$ -GABA_ARs in ethanol consumption and reinforcement

Individual GABA_A receptor (GABA_AR) subunits have been implicated in NAc ethanol reinforcement using rodent models. The GABA_A receptor subunit δ co-assembles preferentially with $\alpha 4$ and β subunits in pentameric receptors with the configuration $\alpha 4\beta\delta$ (Sur *et al.*, 1999a) which are located extrasynaptically (Nusser, Sieghart and Somogyi, 1998). The agonist THIP is preferential for $\alpha 4\beta\delta$ GABA_A receptors (Ebert *et al.*, 1994) and was found to increase ethanol consumption in rats via IP injection (Boyle *et al.*, 1993).

Extrasynaptic $\alpha 4\beta\delta$ receptors are also of particular interest in ethanol consumption as they were found to be the most rapidly upregulated GABA receptors following high-dose or chronic ethanol administration in rats (Liang *et al.*, 2007). These receptors are located extrasynaptically on medium spiny neurons (MSNs) in the NAc where they mediate a tonic inhibition of MSNs (Maguire *et al.*, 2014).

C57Bl/6J mice with a constitutive deletion of the GABA_A δ subunit (δ -KO mice) subunit show reduced ethanol consumption in a continuous-access 'Two-bottle choice' test (Mihalek *et al.*, 2001). Transgenic mice with a deletion of the $\alpha 4$ subunit ($\alpha 4$ -KO mice) show reduced enhancement of GABA_AR mediated tonic currents by THIP and a reduced sensitivity to its motor incoordinating effects

(Chandra *et al.*, 2006) although they have not previously been reported to show reduced ethanol consumption. Mice carrying mutations of the *gabrb1* gene encoding GABA_A subunit $\beta 1$, rendering $\beta 1$ -GABA_ARs tonically active, are less sensitive to the acute motor effects of alcohol and show increased ethanol self-administration associated with enhanced tonic inhibition in the NAc (Anstee *et al.*, 2013).

Within the ventral striatum $\alpha 4$ is notably most highly expressed in the NAc (Schwarzer *et al.*, 2001), an area which has been implicated in mediating the addictive properties of many drugs of abuse (Everitt and Robbins, 2005). Several studies have found that $\alpha 4\beta\delta$ receptors in the NAc shell mediate alcohol consumption and reinforcement. Virally mediated knockdown of δ (Nie *et al.*, 2011) or $\alpha 4$ (Rewal *et al.*, 2009) subunits in the NAc shell, but not core, reduced ethanol consumption by rats during intermittent access in a 'Two-bottle choice' test. Knockdown of $\alpha 4$ in the NAc shell also reduced operant ethanol self-administration in rats (Rewal *et al.*, 2012). Together these phenotypes strongly imply that tonic inhibition via $\alpha 4\beta\delta$ receptors has a major role in the reinforcing effects of alcohol.

Electrophysiological evidence for the effects of ethanol on these receptors is mixed. Early results found that low, ecologically valid doses of ethanol enhanced GABA_A gated current in *Xenopus laevis* oocytes expressing $\alpha 4\beta\delta$ receptors and that $\alpha 4\beta 3\delta$ were uniquely sensitive to doses as low as 1mM (Sundstrom-Poromaa *et al.*, 2002; Wallner, Hancher and Olsen, 2003). This technique was also used to demonstrate that RO15-4513 blocked ethanol enhancement of $\alpha 4\beta\delta$ mediated current at doses that did not reduce GABA gated Cl⁻ current (Wallner, Hancher and Olsen, 2006) suggesting a mechanism for the ability of RO15-4513 to reduce ethanol self-administration (Rassnick *et al.*, 1993). These results are controversial as they are variable and have failed to replicate. Even in experiments which supported this model the ethanol concentration required for similar enhancement of current varied from 1mM (Sundstrom-Poromaa *et al.*, 2002) to 30mM (Wallner, Hancher and Olsen, 2003). Independent experiments on *Xenopus* oocytes and human fibroblasts expressing rat, murine or human GABA_A $\alpha 4/\beta 3/\delta$ subunits reported that high doses of ethanol were required to enhance current; 100mM in oocytes and 300mM in fibroblasts (Borghese *et al.*,

2006). The BAC used to define 'binge drinking' equates to approximately 17mM (NIAAA, 2004) so these results are unlikely to be physiologically relevant.

It is also not clear whether direct or indirect action of ethanol at $\alpha 4\beta\delta$ receptors mediates its acute behavioural effects. Neither δ -KO nor $\alpha 4$ -KO mice displayed different ethanol-related changes in anxiety or sedation when compared to wild-type mice (Mihalek *et al.*, 2001; Chandra *et al.*, 2006) although this may be due to compensatory changes in transgenic mice whereby other voltage gated ion channels can be upregulated to counterbalance changes in neuronal excitability (Brickley *et al.*, 2001). One possible mechanism by which ethanol may enhance GABA_A current is via certain neurosteroids which act as positive allosteric modulators of GABA_A receptors, even at low nanoMolar to microMolar concentrations (Lambert *et al.* 1995; Lambert *et al.* 2003). IP ethanol administration was found to greatly increase levels of the neurosteroid allopregnanolone (ALLOP) in the brain and this increase was much larger in alcohol-preferring relative to non-preferring rats (Barbaccia *et al.*, 1999). Interestingly IP injection of ALLOP has also been found to enhance ethanol consumption during operant self-administration and 'two-bottle choice' procedures in rats (P H Janak, Redfern and Samson, 1998) and C57BL/6J mice (Sinnott, Phillips and Finn, 2002) respectively.

Previous experiments examining the role of $\alpha 4$ in ethanol consumption (described above) have primarily focused on two-bottle choice or operant self-administration. We wished to expand these studies using a DID protocol as under these conditions mice typically consume more ethanol and achieve higher BACs (Crabbe, Harris and Koob, 2011). This would be of interest as it may discover a role of $\alpha 4$ containing receptors in binge-like drinking specifically. Prior studies have also largely been conducted in rats therefore replication of these effects in murine models would provide convergent evidence from two species, which is more robust, and enables higher through-put experiments to which mice are more suited.

Here we used the DID procedure to investigate the role $\alpha 4$ -containing GABA_ARs play mediating ethanol consumption in mice. We compared transgenic C57BL/6J mice with a deletion of the *gabra4* gene encoding the $\alpha 4$ subunit ($\alpha 4$ -KO mice)

to wild-type ($\alpha 4$ -WT) and heterozygous ($\alpha 4$ -Het) littermates (described in Chapter 2).

IP injection of THIP has been found to modulate ethanol consumption but with mixed results (Boyle et al. 1993, Ramaker et al. 2012). This may be due to the sedative effect of THIP at higher doses (Ebert *et al.*, 1994). We administered THIP intraperitoneally at a range of doses (0, 4, 8, 16mg/kg) to $\alpha 4$ -KO, $\alpha 4$ -WT and $\alpha 4$ -Het littermates directly prior to the drinking period to see whether THIP would affect ethanol consumption and whether deletion of $\alpha 4$ would disrupt this.

In previous experiments we infused THIP directly into the NAc of wild-type C57BL/6J mice prior to the drinking period. This increased drinking relative to control sessions where saline was infused (Macpherson, Stephens and King, unpublished data). Here we attempt to replicate this study using $\alpha 4$ -KO and $\alpha 4$ -WT mice to confirm whether $\alpha 4$ -GABA_ARs are required for intra-NAc THIP to increase drinking.

The effects of these receptors may also depend on expression within different neuronal classes and subtypes. MSNs throughout the basal ganglia, including the NAc, can be classified as 'D1' or 'D2' based on their expression of dopamine D1 or D2 receptors respectively (Gerfen *et al.*, 1990). The D1 and D2 MSN pathways appear to have different and opposing roles mediating the rewarding properties of drugs (reviewed in Chapter 1/2 and Lobo and Nestler, 2011).

Deletion of either D1 or D2 type dopamine receptors in reduces ethanol consumption in transgenic mice (El-Ghundi *et al.*, 1998; Phillips *et al.*, 1998). Multiple experiments have found that systemically D1 and D2 agonists reduce ethanol preference and consumption (Linseman, 1990; Silvestre *et al.*, 1996; Cohen, Perrault and Sanger, 1999).

D1 antagonists or agonists injected into the NAc-shell but not core reduced or enhanced operant responding for ethanol respectively (Hauser *et al.*, 2015) indicating that D1 activity in the NAc enhances motivation for ethanol. Intra-NAc injection of D2 antagonists also reduced responding for ethanol and this effect was enhanced by co-administration of D1 agonists suggesting that D1-like and D2-like receptors in the NAc interact in the regulation of ethanol self-administration (Clyde W. Hodge, Samson and Chappelle, 1997). In the same

study moderate doses of the D2 agonist quinpirole (1 μ g) increased total responses and response rate, whereas higher doses (4 to 10 μ g) decreased total responding due to early termination. Co-administration of either D1 agonists or antagonists prevented the enhanced responding observed with the lower doses of quinpirole which suggests that an intermediate level of D1 activation is required to observe the D2 effect.

D1 and D2 MSNs in the NAc also undergo different physiological adaptations following chronic alcohol consumption. Two weeks of daily DID sessions shifted D1 MSNs in the NAc from a state of Long Term Depression (LTD) to Long Term Potentiation (LTP) and did the opposite to D2 MSNs shifting them from LTP to LTD when compared to naive mice (Ji *et al.*, 2017). Repeated ethanol drinking in a limited-access 'two-bottle-choice' upregulated synaptic glutamate transmission in striatal D1 neurons and inhibitory synaptic GABA transmission in D2 neurons (Cheng *et al.*, 2017). This study further demonstrated that inhibition of D1-MSNs or excitation of D2-MSNs using DREADDs attenuated excessive alcohol consumption. Together these results indicate that D1 activation or D2 inactivation in the striatum promotes drinking and this pattern of activity is reinforced by maladaptive changes in D1 and D2 MSNs.

The Cre-lox recombination system allows conditional knock-out of a gene by crossing an animal carrying a 'floxed' allele with one expressing Cre under a desired promoter (Gu *et al.*, 1994, see Chapters 1 & 2). We have used this technique to generate conditional knockout of the $\alpha 4$ subunit in D1 or D2 expressing neurons in C57BL6J mice (see chapter 2). In a cocaine-conditioned place preference (CPP) experiment constitutive $\alpha 4$ knockout mice did not differ from WT mice however conditional knock-out of $\alpha 4$ in D1 MSNs increased CPP whilst both constitutive and D2 MSN specific knockout enhanced conditioned reinforcement (Maguire *et al.*, 2014; Macpherson *et al.*, 2016).

As $\alpha 4$ appears to mediate different effects on cocaine conditioned behaviour via the D1 and D2 MSN pathways we wished to examine whether D1 or D2 specific manipulations of $\alpha 4\beta\delta$ receptors would differentially affect behaviours related to ethanol as well. We compared $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO with their $\alpha 4$ -D1-WT and $\alpha 4$ -D2-WT littermates (described in Chapter 2) in a standard DID experiment.

3.2. Materials and Methods

3.2.1. Animals

All mice were generated on C57BL/6J background strain. Mice homozygous for a null-mutation of the *gabra4* gene ($\alpha 4$ -KO) and homozygous wild-type mice ($\alpha 4$ -WT) were generated by breeding heterozygous mice carrying one copy each of both the knock-out and wild-type allele ($\alpha 4$ -Het) as described previously in chapter 2 (Chandra *et al.*, 2006; Maguire *et al.*, 2014).

As described in chapter 1 we produced conditional knock-out lines we crossed 'floxed' $\alpha 4$ mice [strain name; B6.129-Gabra4tm1.2Geh/J; Jackson Laboratory] (Chandra *et al.*, 2006) with BAC D1-CRE [MMRRC strain B6.FVB(Cg)-Tg(Drd1a-cre)EY266Gsat/Mmucd] or BAC D2-CRE [MMRRC strain B6.FVB(Cg)-Tg(Drd2-cre)ER44Gsat/ Mmucd] (Gong *et al.*, 2007) to result in cre-mediated deletion of $\alpha 4$ in either D1 or D2 expressing cells of the offspring. Mice hemizygous for the BAC D1-Cre transgene and homozygous for the 'floxed' $\alpha 4$ transgene were bred with homozygous 'floxed' $\alpha 4$ mice to produce $\alpha 4$ -D1-KO and $\alpha 4$ -D1-WT littermates whilst mice hemizygous for the BAC D2-Cre transgene and homozygous for the 'floxed' $\alpha 4$ transgene were bred with homozygous 'floxed' $\alpha 4$ mice to produce $\alpha 4$ -D2-KO and $\alpha 4$ -D2-WT littermates as previously described (Maguire *et al.*, 2014).

Male and female mice weighing between 20-30g and aged between 2-4 months, were removed from groups of 2-3 and housed separately 7 days prior to experiments, with food and water available *ad libitum*. During the habituation period and experiment a reversed 12hr light/dark cycle was used (lights on at 11:00 P.M.) with holding room temperature maintained at $21 \pm 2^\circ\text{C}$ and humidity $50 \pm 5\%$.

3.2.2. Drugs

THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) was kindly donated by Bjarke Ebert (Lundbeck, Valby, Denmark). For IP injections THIP was dissolved in 0.9% saline to the required concentrations and administered at a volume of 10ml/kg. Active doses for intracranial infusions were selected based on previous data. THIP was infused bilaterally at a concentration of 3mM and a volume of 0.5 μ l per side. DS2 (4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl

benzamide) was dissolved in vehicle of 2% Tween-20 and 2% DMSO in 0.9% saline to a concentration of 0.3mM and infused bilaterally a volume of 0.5ul per side.

3.2.3. Drinking in the Dark procedure

Habituation phase: mice were singly housed and acclimatised to the reverse 12hr dark/light cycle for 7 days. To habituate mice to experimental conditions over the following 5 days water bottles were replaced for 2 hours each day, at 3 hours after lights-off, with sippers (North Kent Plastics, Kent, UK) attached to 10ml serological pipettes, containing tap water, to allow us to record the volume of fluid consumed in each session.

Test Phase: over 5 days with mice received daily 2-hour drinking sessions starting at 3 hours after lights-off. On days 1 and 2 water bottles were replaced with pipettes containing tap water. On days 3 to 5 water bottles were replaced with pipettes containing a 15% ethanol solution. This was selected as in pilot studies 15% was the highest concentration which did not reduce drinking due to taste aversion (data not shown). Consumption was recorded at the end of each session.

α 4-KO vs Heterozygote and WT littermates: Groups of naïve male and female α 4-WT (n=7), α 4-Het (n=7) and α 4-KO (n=7) littermates were compared in the standard DID procedure. The number of males and females was balanced between groups (5 males, 2 females).

α 4-D1-KO vs α 4-D1-WT littermates: Groups of naïve male and female α 4-D1-KO (n=7), and α 4-D1-WT (n=7) littermates were compared in a standard DID procedure. The number of males and females was balanced between groups (4 males, 3 females).

α 4-D2-KO vs α 4-D2-WT littermates: Groups of naïve male and female α 4-D2-KO (n=7), and α 4-D2-WT (n=7) littermates were compared in a standard DID procedure. The number of males and females was balanced between groups (4 males, 3 females).

3.2.4. IP THIP administration dose response

Following 2 days resting period the same mice were given IP injections of 0.9% saline at 3 hours after lights off over 5 days habituation, followed by another 2 days resting period. The mice then underwent DID sessions as described above over the course of 8 days receiving IP injections directly prior to the drinking period. THIP was dissolved in 0.9% saline and administered at doses of 0, 4, 8 and 16mg/kg and a volume of 10ml/kg. Doses were allocated in a Latin-square design so that each dose was given to at least one animal per day and animals received all doses prior to one water-available session and prior to one ethanol-available session.

3.2.5. Stereotaxic Cannulation

Mice anaesthetised with isoflurane were implanted stereotaxically with bilateral guide cannulae (26 ga., 10mm) aimed at the NAc (coordinates AP1.34; L+/- 1.00; DV -3.20, Paxinos and Franklin, 2001). Following surgery, mice were singly housed and underwent a one-week recovery/habituation period.

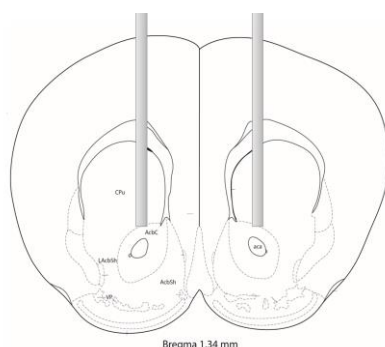


Figure 3.1. Target site for infusions of drug via indwelling cannulae. Coordinates AP1.34; L+/- 1.00; DV -3.20, from Bregma.

3.2.6. Intra-NAc Infusions

On alternate days mice were given an infusion of drug or saline to the NAc. A steel infuser (33ga., 11mm) connected via polyvinyl tubing to a (5µl) Hamilton Gastight syringe was used to infuse 0.5µl of either saline, THIP (3 mM) or DS2 (0.03mM) bilaterally over 90 seconds and left to settle for 90 seconds before infusers were removed.

3.2.7. Histological Confirmation of Placements

Location of cannulae was confirmed histologically following experiment. India ink (Windsor and Newton, UK) was infused bilaterally at a volume of 0.5 μ l. Mice were euthanised by IP injection of Sodium Pentobarbital (10ml/kg). Mice brains were perfused via the aorta with 25ml (5 minutes of 5ml/min) of phosphate buffer solution (PBS) followed by 75ml (15 minutes of 5ml/min) of 4% paraformaldehyde (PF) (Sigma-Aldrich, St. Lois, MO, USA) in PBS. After perfusion cannulae were removed then brains were extracted and post-fixed overnight in 4% PF in PBS at 4°C, then transferred into 30% sucrose solution in PBS and left for 3 days at 4°C to cryoprotect. Coronal sections (30 μ m thick) were cut using a cryostat and collected in PBS-azide.

Sections were mounted to Superfrost slides (Thermofisher, UK) and air dried overnight. Sections were immersed in distilled water for 2 times 10 minutes then immediately dehydrated in an ethanol series immersed in 30% followed by 60%, 90%, 95% and 100% ethanol for 2 minutes each then in clearing solution (Histoclear, National Diagnostics) for 10 minutes. Sections were re-hydrated in 100% ethanol followed by distilled water for 2 minutes each then immersed in 0.1% Cresyl Violet in 0.1M Sodium Acetate, 0.1M Acetic acid buffer solution for 10 minutes. Slides were rinsed in distilled water followed by 70% ethanol then dehydrated in 30% followed by 60%, 90%, 95% and 100% ethanol for 2 minutes each then in clearing solution (Histoclear, National Diagnostics) for 10 minutes. Coverslips were applied using mounting medium (Histomount, National Diagnostics).

3.2.8. Stereotaxic Viral Infusion

C57BL/6J mice anaesthetised with isoflurane were stereotaxically infused with AAV-Cre-mCherry or AAV-GFP bilaterally into the NAc (coordinates AP1.34; L+/-1.40; DV -4.20, (Paxinos and Franklin, 2001)). A steel infuser (33ga) connected via polyvinyl tubing to a (5 μ l) Hamilton Gastight syringe was used to infuse 1 μ l (0.5 μ l per side) of virus (1×10^9 IU/ml) at a rate of 0.2 μ l/min for 5 minutes, then left to settle for an additional 5 minutes. Following surgery mice were singly housed and allowed to recover for 7 days. Viruses contained mCherry and GFP fluorescent tags respectively which allowed histological confirmation of the targeted area.

We used 17 'floxed' $\alpha 4$ mice; 8 receiving infusion of AAV-Cre and 9 receiving infusion of AAV-GFP. Of these we excluded 1 AAV-Cre and 2 AAV-GFP from analysis due to inaccurate placement resulting in $n=7$ per group.

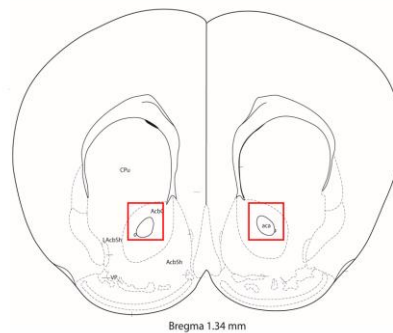


Figure 3.2. Target site for infusions of AAV-Cre or AAV-GFP virus. Coordinates AP1.34; L+/- 1.4; DV -4.20, from Bregma.

3.2.9 Immunohistochemistry

Following experiments mice were euthanised by IP injection of Sodium Pentobarbital (10ml/kg). Mice brains were perfused via the aorta with 25ml (5 minutes of 5ml/min) of phosphate buffer solution (PBS) followed by 75ml (15 minutes of 5ml/min) of 4% paraformaldehyde (PF) (Sigma-Aldrich, St. Lois, MO, USA) in PBS. After perfusion, brains were removed and post-fixed overnight in 4% PF in PBS at 4°C, then transferred into 30% sucrose solution in PBS and left for 3 days at 4°C to cryoprotect. Coronal sections (30 μ m thick) were cut using a cryostat and collected in PBS-azide.

Free floating sections were washed 3 times in PBS for 10 minutes then incubated in blocking solution, 3% Normal Goat Serum (Vectorlabs) in PBS-T, for 1 hour with gentle agitation. Sections were then immediately incubated overnight in rabbit anti-GFP ployclonal primary antibody (1:10,000, Abcam ab6556) or rabbit anti-mCherry antibody (1:10,000, Abcam ab167453) diluted in blocking solution at 4°C. Sections were washed 3 times in PBS for 10 minutes before incubation in fluorescently tagged Alexafluor 488/568 anti-rabbit secondary antibodies (1:600, Thermofisher) diluted in blocking solution for 2 hours at room temperature.

3.2.10. Statistics

All statistical analysis was carried out using IBM SPSS software v.24. All analyses were initially carried out including sex as an independent variable however we found no main effects or interactions with other variables and sex was therefore excluded in the presented analyses for clarity.

3.2.10.1 Standard DID procedures

Prior to the test phase the starting weight of mice was taken and we compared this between genotypes using a one-way ANOVA to ensure weight was similar between experimental groups.

The mean volume of water or ethanol consumed was calculated from 2 and 3 sessions respectively. Ethanol consumption as a function of mouse weight (g/kg) was calculated and averaged over 3 days.

Mean fluid consumption (ml) of water and ethanol by each genotype was compared using a one-way repeated measures ANOVA. Mean ethanol consumption (g/kg) of each genotype was compared by one-way ANOVA and followed by a Tukey's Least Square Difference (LSD) post hoc test where there were more than two groups.

3.2.10.2 IP THIP administration

Volume of water or ethanol solution consumption at each dose was averaged and compared between genotypes using multi-factor repeated-measures ANOVA where genotype was a between subjects factor.

Ethanol consumption (g/kg) at each dose was averaged for each genotype and compared in a repeated-measures ANOVA where genotype was a between subjects factor.

3.2.10.3 Intra-NAc THIP administration

The volume of water or ethanol consumed by $\alpha 4$ -WT and $\alpha 4$ -KO mice administered either saline or THIP was averaged from 2 sessions per condition. Volume drunk was compared using a multi-factor repeated measures ANOVA where Drug-Treatment and Solution (water or ethanol) were the factors and genotype was the between-subjects variable. Ethanol consumption as a function

of bodyweight (g/kg) for each condition was compared using a repeated measures ANOVA.

Similarly ethanol consumed by $\alpha 4$ -WT and $\alpha 4$ -KO mice administered either Vehicle or DS2 was averaged from 2 sessions per condition. Volume drunk was compared using a multi-factor repeated measures ANOVA where Drug-Treatment and Solution (water or ethanol) were the factors and genotype was the between-subjects variable. Ethanol consumption as a function of bodyweight (g/kg) for each condition was compared using a repeated measures ANOVA.

3.3. Results

3.3.1. *Gabra4* deletion reduces ethanol consumption in drinking in the dark

We compared $\alpha 4$ -WT, $\alpha 4$ -Het and $\alpha 4$ -KO mice in a standard DID experiment in which we measured their water and ethanol consumption.

$\alpha 4$ -KO animals consumed less ethanol but similar volume of water to $\alpha 4$ -WT and $\alpha 4$ -Het littermates. All genotypes drank a lower volume of ethanol than water ($F_{(1,18)} = 63.4$, $p < 0.001$). There was a significant interaction between genotype and solution ($F_{(2,18)} = 2.6$, $p < 0.05$). The post hoc test revealed $\alpha 4$ -KO mice drank significantly less ethanol (mean = 0.176, S.E. = 0.04, $n = 7$) than their $\alpha 4$ -WT (mean = 0.326, S.E. = 0.04, $n = 7$) or $\alpha 4$ -Het (mean = 0.331, S.E. = 0.04, $n = 7$) littermates, $p < 0.05^*$. We found no significant difference in starting-weight between genotypes ($F_{(2,18)} = 0.46$, $p < 0.5$).

Ethanol consumption as a function of mouse weight (g/kg) was significantly lower in $\alpha 4$ -K) mice compared to $\alpha 4$ -WT and $\alpha 4$ -Het littermates. We found a significant main effect of genotype ($F_{(2,18)} = 3.78$, $p < 0.05$). The post hoc test found that $\alpha 4$ -KO mice consumed significantly less ethanol (mean = 0.929, S.E. = 0.25, $n = 7$) than their $\alpha 4$ -WT (mean = 1.65, S.E. = 0.04, $n = 7$) or $\alpha 4$ -Het (mean = 1.55, S.E. = 0.04, $n = 7$) littermates, $p < 0.05^*$.

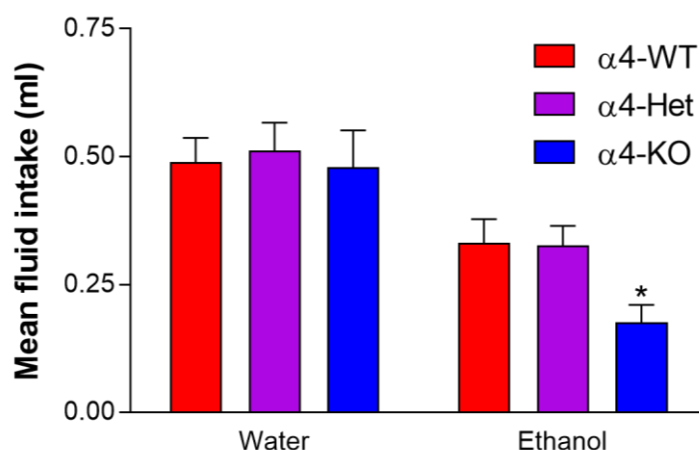


Figure 3.3 α4-KO (n=7) drank significantly less 15% ethanol solution than their α4-WT (n=7) or α4-Het (n=7) littermates $p < 0.05^*$, however water consumption was similar between genotypes. Error bars represent SEM.

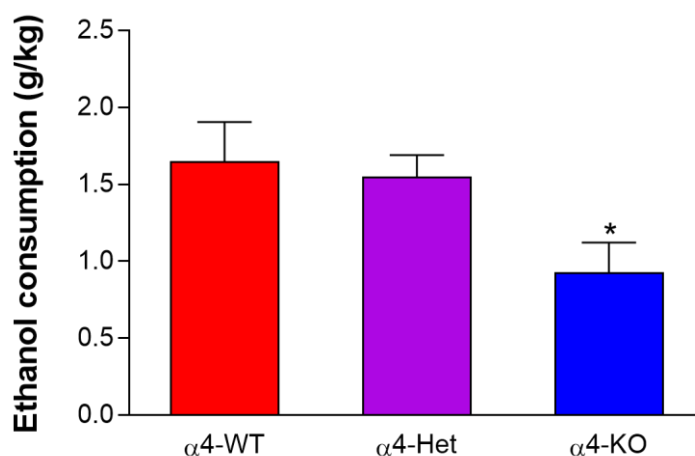


Figure 3.4 α4 knockout mice (n=7) consumed significantly less ethanol as a function of bodyweight than their wild-type (n=7) or heterozygous (n=7) littermates, $p < 0.05^*$. Error bars represent SEM.

3.3.2. IP THIP administration reduces both water and ethanol consumption in DID in α4-WT but not α4-KO mice

We administered THIP intraperitoneally at various doses to α4-WT, α4-Het and α4-KO mice prior to drinking sessions in a DID experiment in which we measured their water and ethanol consumption under each dose.

At low doses (0,4,8mg/kg) IP THIP had no effect on either genotype however at 16mg/kg IP THIP reduced consumption of both water and ethanol in $\alpha 4$ -WT and $\alpha 4$ -Het mice. In contrast $\alpha 4$ -KO mice were unaffected by THIP although they drank significantly less ethanol at baseline.

There was a significant main effect of THIP dose on water consumption ($F_{(3,18)} = 8.797$, $p < 0.001$) and a significant interaction between genotype and THIP dose on water consumption ($F_{(3,18)} = 2.6$, $p < 0.05$) whereby the volume drunk was reduced at high doses in $\alpha 4$ -WT and $\alpha 4$ -Het but not reduced to the same extent $\alpha 4$ -KO mice. There was a significant main effect of THIP dose on 15% ethanol consumption ($F_{(3,18)} = 15.5$, $p < 0.001$) and a significant interaction between genotype and THIP dose on 15% ethanol consumption ($F_{(3,18)} = 2.3$, $p < 0.05$) whereby the volume drunk was greatly reduced at 16mg/kg THIP in $\alpha 4$ -WT and $\alpha 4$ -Het but not to the same extent $\alpha 4$ -KO mice.

Similarly, at low doses (0,4,8mg/kg) IP THIP had no effect on ethanol consumption as a function of bodyweight (g/kg) in either genotype however at 16mg/kg IP THIP reduced ethanol consumption in $\alpha 4$ -WT and $\alpha 4$ -Het but not to the same extent $\alpha 4$ -KO mice. There was a significant main effect of THIP dose ($F_{(3,18)} = 14.243$, $p < 0.001$) and a significant interaction between genotype and THIP dose ($F_{(3,18)} = 2.4$, $p < 0.05$) whereby the ethanol consumption was reduced at high doses in $\alpha 4$ -WT and $\alpha 4$ -Het but not reduced to the same extent $\alpha 4$ -KO mice.

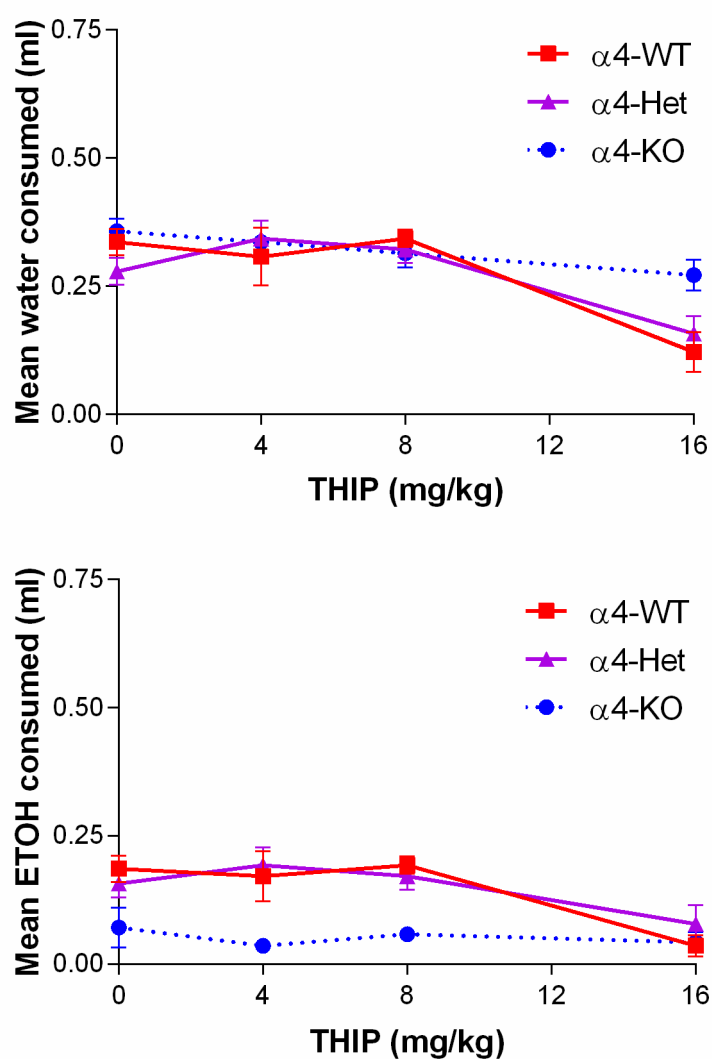


Figure 3.5 A) Volume of water consumption decreased in all genotypes at 16mg/kg THIP ($n=21$, $p<0.05$). B) Volume of ethanol consumption was decreased by 16mg/kg THIP in WT ($n=7$) and Heterozygous ($n=7$) and to some lesser extent $\alpha 4$ -KO mice ($n=7$) $p<0.05$. Error bars represent SEM.

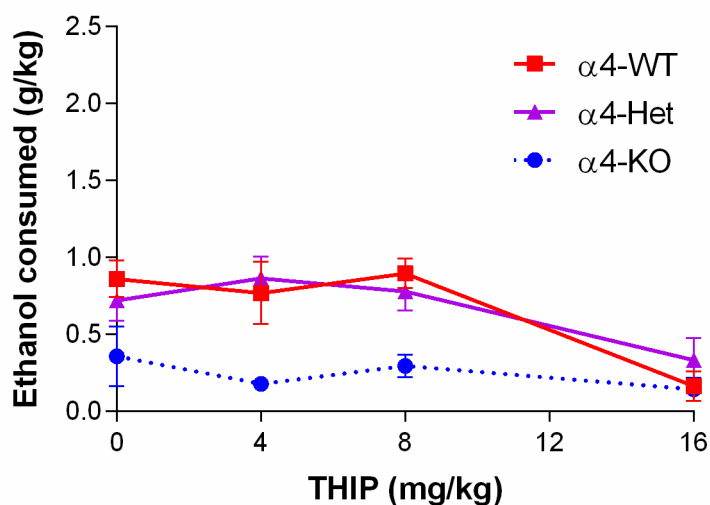


Figure 3.6 α4-KO mice (n=7) consumed significantly less ethanol as a function of bodyweight than their wild-type (n=7) or heterozygous (n=7) littermates, $p < 0.05$. Ethanol consumption was decreased by 16mg/kg THIP in WT (n=7) and Heterozygous (n=7), but not α4-KO mice (n=7) $p < 0.05$. Error bars represent SEM.

3.3.3 Effect of Intra-NAc THIP on water and ethanol consumption in α4-KO and α4-WT mice

We administered THIP or saline directly to the NAc of α4-WT and α4-KO mice prior to drinking sessions in a DID experiment in which we measured their water and ethanol consumption under each treatment.

Both genotypes drank significantly less ethanol than water ($F_{(1,14)} = 94.03$, $p < 0.01$) whilst α4-KO mice drank a similar amount of water but significantly less ethanol than α4-WT littermates ($F_{(1,14)} = 5.914$, $p < 0.05$). We did not find a significant effect of drug ($F_{(1,14)} = 1.42$, $p = 0.254$; NS) or a significant drug by solution ($F_{(1,14)} = 0.243$, $p = 0.629$; NS) or drug by genotype ($F_{(1,14)} = 0.008$, $p = 0.938$; NS) interaction. The drug by solution by genotype interaction showed a trend whereby THIP increased volume of ethanol consumed in α4-WT but not α4-KO animals however this was non-significant ($F_{(1,14)} = 4.293$, $p = 0.057$).

Ethanol consumption (g/kg) was significantly lower in α4-KO than α4-WT mice ($F_{(1,14)} = 67.9$, $p < 0.001$). We found no main effect of drug ($F_{(1,14)} = 0.638$, $p = 0.438$; NS) or drug by solution interaction ($F_{(1,14)} = 2.56$, $p = 0.131$) indicating that THIP did not significantly reduce ethanol consumption.

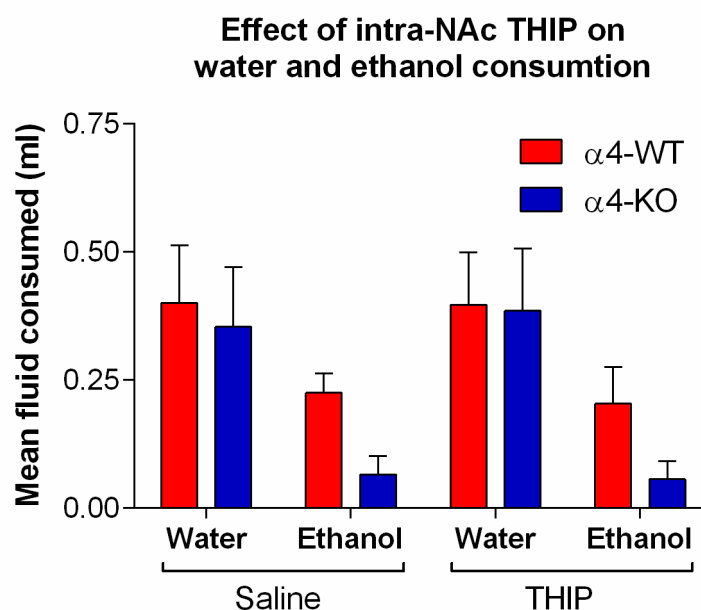


Figure 3.7. We found no effect of intra-NAc THIP on either water or ethanol consumed in either genotype. Error bars represent SEM.

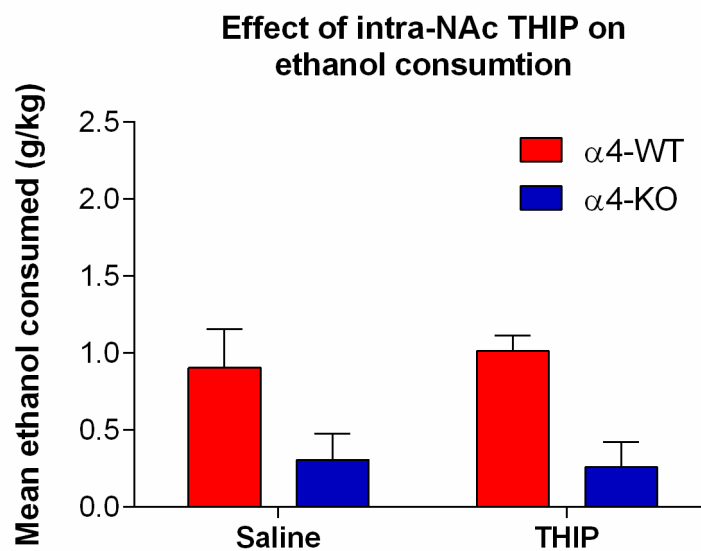


Figure 3.8 We found no effect of Intra-NAc THIP on ethanol consumption as a function of bodyweight (g/kg) in either genotype. Error bars represent SEM.

3.3.4. Effect of Intra-NAc DS2 on water and ethanol consumption in $\alpha 4$ KO and $\alpha 4$ -WT mice

We administered DS2 or vehicle directly to the NAc of $\alpha 4$ -WT and $\alpha 4$ -KO mice prior to drinking sessions in a DID experiment in which we measured their water and ethanol consumption under each treatment.

Both genotypes drank significantly less ethanol than water ($F_{(1,14)} = 97.53$, $p < 0.01$) whilst $\alpha 4$ -KO mice drank a similar amount of water but significantly less ethanol than $\alpha 4$ -WT littermates ($F_{(1,14)} = 5.53$, $p < 0.05$). We did not find a significant effect of drug ($F_{(1,14)} = 1.074$, $p = 0.318$; NS) or a significant drug by solution ($F_{(1,14)} = 1.074$, $p = 0.318$; NS) or drug by genotype ($F_{(1,14)} = 2.104$, $p = 0.169$; NS) interaction. The drug by solution by genotype interaction showed a trend whereby DS2 increased volume of ethanol consumed in $\alpha 4$ -WT but not $\alpha 4$ -KO animals however this was non-significant ($F_{(1,14)} = 2.104$, $p = 0.169$).

We found no main effect of drug on ethanol consumption (g/kg) ($F_{(1,14)} = 1.274$, $p = 0.278$; NS) or drug by solution interaction ($F_{(1,14)} = 2.22$, $p = 0.158$; NS) indicating that THIP did not significantly reduce ethanol consumption.

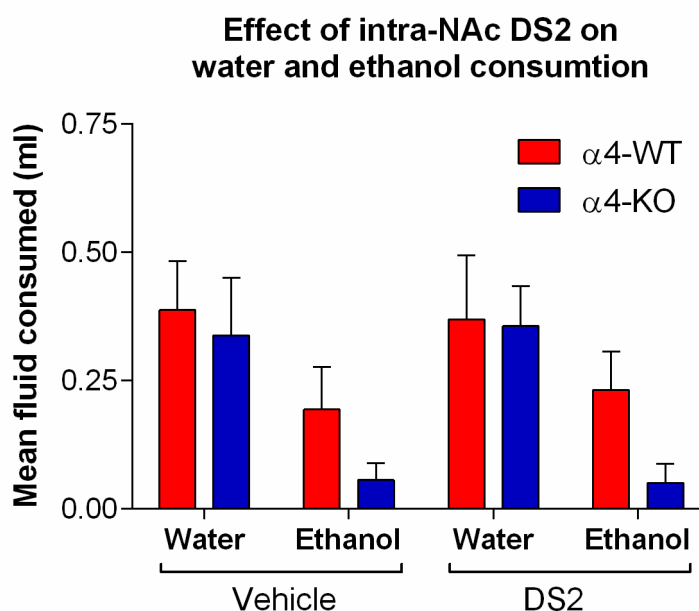


Figure 3.9. We found no effect of intra-NAc DS2 on either water or ethanol consumed in either genotype. Error bars represent SEM.

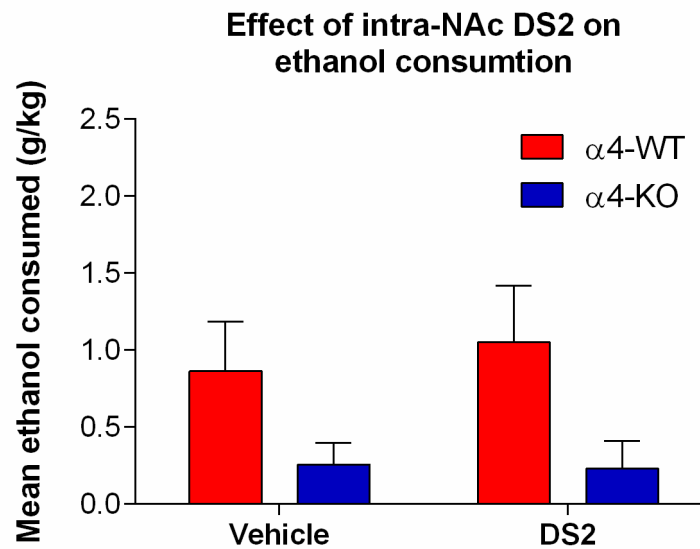


Figure 3.10. We found no effect of intra-NAc DS2 on ethanol consumption as a function of bodyweight (g/kg) in either genotype. Error bars represent SEM.

3.3.5. Effect of selective knockout of $\alpha 4$ in NAc on ethanol consumption

In order to examine the effects of reduced $\alpha 4$ expression, specifically in the NAc, on ethanol consumption we used an AAV viral vector to express Cre in the NAc of 'floxed' $\alpha 4$ mice to knockdown $\alpha 4$. We compared these mice with 'floxed' $\alpha 4$ mice which received a control AAV virus in a standard DID test.

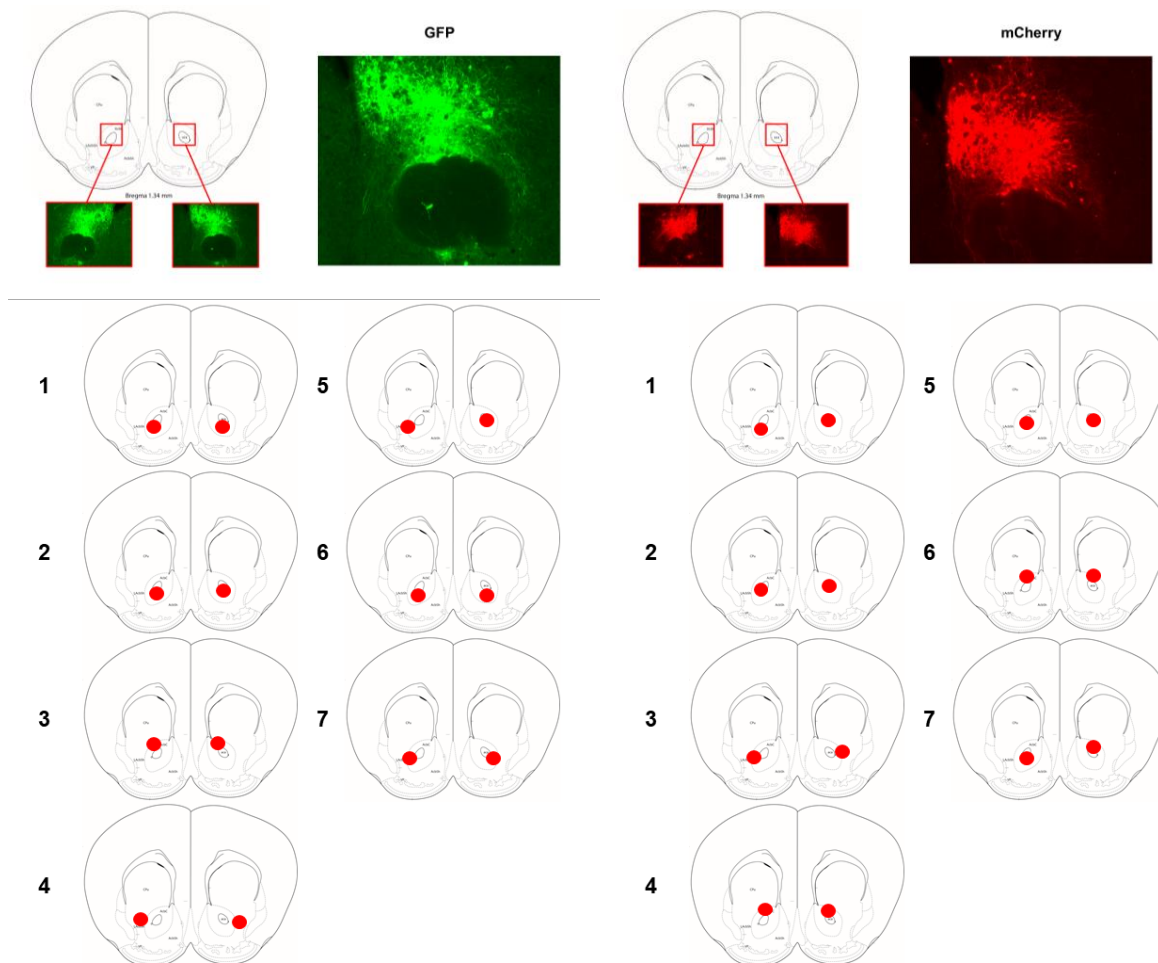


Figure 3.11. Example of viral expression in the NAc of experimental animal at target site (upper panels) and diagram indicating actual injection sites for each animal (lower panels).

We found no significant difference in starting-weight between virus groups ($F_{(1,12)} = 0.029$, $p = 0.87$; NS). Both groups drank a lower volume of 15% ethanol than water ($F_{(1,12)} = 32.053$, $p < 0.001$). There was a significant interaction between virus and solution ($F_{(2,18)} = 10.283$, $p < 0.05$) as AAV-Cre treated mice drank less 15% ethanol solution than AAV-GFP treated mice. Ethanol consumption as a function of mouse weight (g/kg) was compared and we found a significant main effect of virus ($F_{(2,18)} = 12.13$, $p < 0.005$).

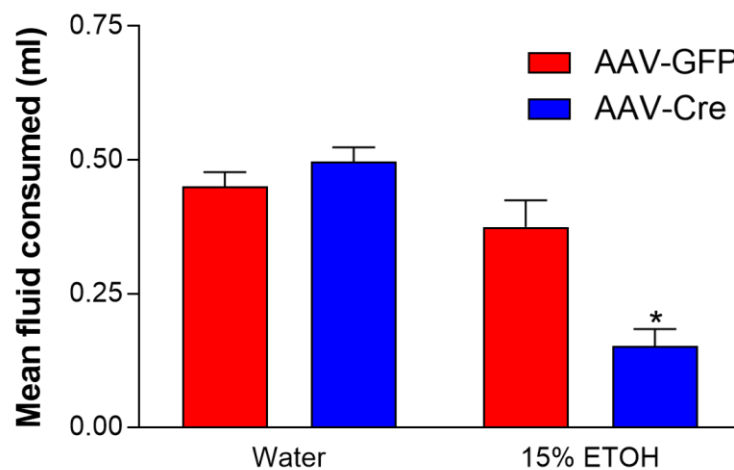


Figure 3.12. AAV-Cre mice (n=7) drank significantly less 15% ethanol than AAV-GFP treated mice (n=7) $p < 0.05^*$. Error bars represent SEM.

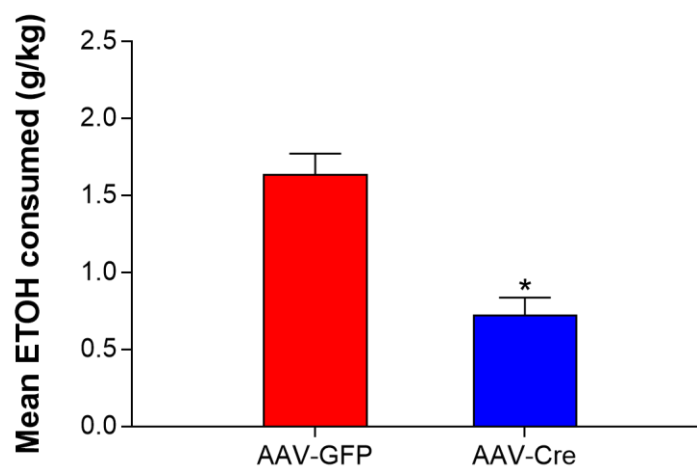


Figure 3.13. AAV-Cre mice (n=7) consumed significantly less ethanol as a function of bodyweight than AAV-GFP treated mice (n=7) $p < 0.005^*$. Error bars represent SEM.

3.3.6. D1 specific deletion of $\alpha 4$ does not affect ethanol consumption in DID

We compared $\alpha 4$ -D1-WT and $\alpha 4$ -D1-KO conditional knockout mice in a standard DID experiment in which we measured their water and ethanol consumption.

We found no significant difference in starting-weight between genotypes ($F_{(1,14)} < 0.001$, $p = 0.992$; NS). Both genotypes drank a lower volume of 15% ethanol than water ($F_{(1,14)} = 52.9$, $p < 0.001$). There was no significant interaction

between genotype and solution ($F_{(1,14)} = 1.02$, $p = 0.33$; NS) indicating both genotypes drank a similar amount of ethanol and water.

Ethanol consumption as a function of mouse weight (g/kg) was compared and we found no significant effect of genotype ($F_{(1,14)} = 0.387$, $p = 0.54$; NS).

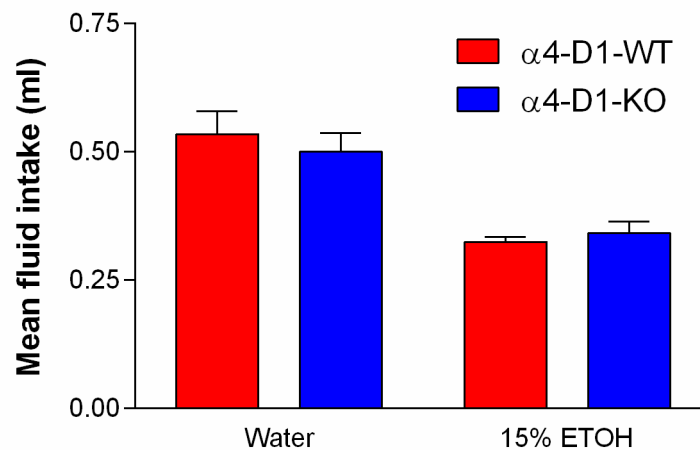


Figure 3.14. Both α4-D1-WT (n=8) and α4-D1-KO (n=8) drank significantly less 15% ethanol than water $p < 0.001$ and drank similar amounts of both water and 15% ethanol. Error bars represent SEM.

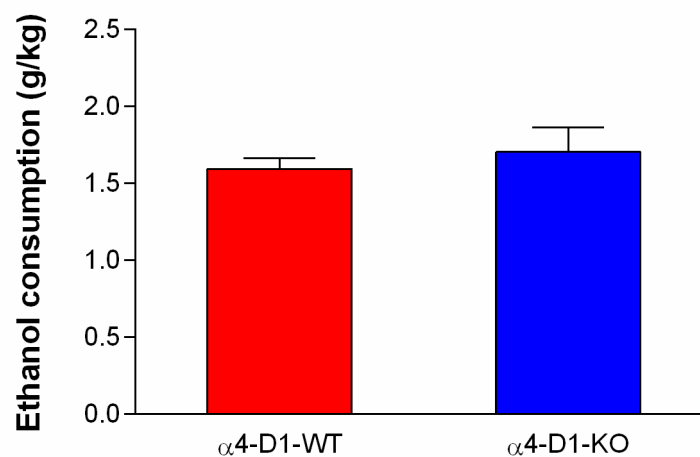


Figure 3.15. α4-D1-WT (n=8) and α4-D1-KO (n=8) consumed a similar amount of ethanol as a function of bodyweight (g/kg). Error bars represent SEM.

3.3.7. D2 specific deletion of $\alpha 4$ does not affect ethanol consumption in DID

We compared $\alpha 4$ -D1-WT and $\alpha 4$ -D1-KO conditional knockout mice in a standard DID experiment in which we measured their water and ethanol consumption.

We found no significant difference in starting-weight between genotypes ($F_{(1,14)} = 0.4$, $p = 0.537$; NS). Both genotypes drank a lower volume of 15% ethanol than water ($F_{(1,14)} = 232.22$, $p < 0.001$). There was no significant interaction between genotype and solution ($F_{(1,14)} = 1.457$, $p = 0.247$; NS) indicating both genotypes drank a similar amount of ethanol and water.

Ethanol consumption as a function of mouse weight (g/kg) was compared and we found no significant effect of genotype ($F_{(1,14)} = 0.065$, $p = 0.802$; NS).

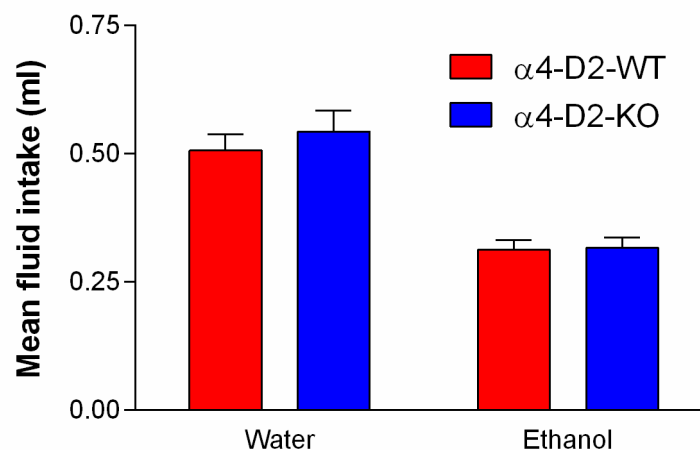


Figure 3.16 Both $\alpha 4$ -D2-WT ($n=8$) and $\alpha 4$ -D2-KO ($n=8$) drank significantly less 15% ethanol than water $p < 0.001$ and drank similar amounts of both water and 15% ethanol. Error bars represent SEM.

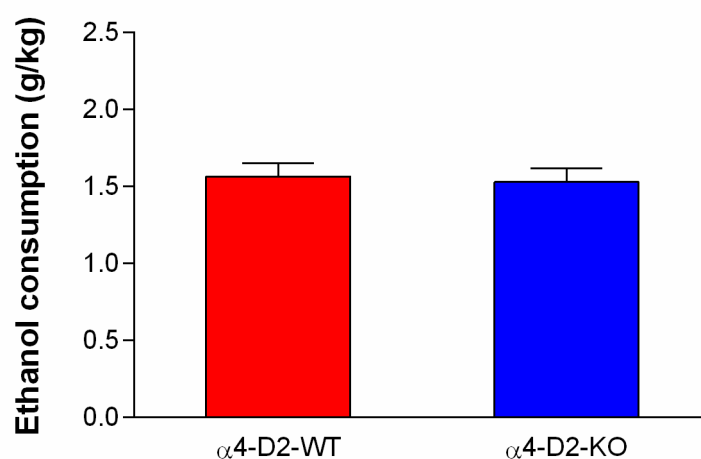


Figure 3.17 $\alpha 4$ -D2-WT (n=8) and $\alpha 4$ -D2-KO (n=8) consumed a similar amount of ethanol as a function of bodyweight (g/kg). Error bars represent SEM.

3.4. Discussion

Several previous studies using rats have found that downregulation of extrasynaptic $\alpha 4\beta\delta$ receptors in the Nucleus Accumbens reduces ethanol self-administration (Rewal *et al.*, 2009, 2012; Nie *et al.*, 2011). We have demonstrated a role of $\alpha 4$ -GABA_ARs in mediating binge-like alcohol consumption in mice. Our data indicate that $\alpha 4$ -GABA_ARs are necessary for the high level of alcohol consumption seen in C57-BL/6J mice as constitutive deletion of $\alpha 4$ was sufficient to reduce alcohol consumption in DID.

In order to test whether the effect of $\alpha 4$ expression on DID was dose-dependent we included $\alpha 4$ -Het mice in this study since they express $\alpha 4$ at ~50% relative to $\alpha 4$ -WT littermates (Chandra *et al.*, 2006; Macpherson, 2013, see Chapter 2). $\alpha 4$ -Het mice did not show any difference in ethanol consumption when compared to $\alpha 4$ -WT littermates. This may be explained by the remaining $\alpha 4$ 50% expression in combination with developmental compensation in constitutive knockouts (Brickley *et al.*, 2001) or it is possible that the threshold for a measurable behavioural effect lies between 50% and 60% knockdown. In prior studies RNAi of $\alpha 4$ in the NAc in rats reduced ethanol consumption where efficiency of the knockdown was ~25% mRNA and ~40% protein levels relative to control (Rewal *et al.*, 2009). Indeed, our manipulation using AAV-Cre virus in the NAc of 'floxed- $\alpha 4$ ' produces a ~60% knockdown (see Chapter 2) and this was sufficient to significantly reduce ethanol consumption in our experiment (Fig 3.12).

Our finding that knockdown of $\alpha 4$ in the NAc reduces ethanol consumption agrees with those similar studies which used RNAi interference of $\alpha 4$ although we used a different species, viral-knockdown method and behavioural tests. By replacing 'Two-Bottle choice' tests with the DID procedure, which usually results in higher BACs, we modelled binge-like drinking behaviour rather than 'moderate drinking' (Rewal *et al.*, 2009; Crabbe, Harris and Koob, 2011). Using this method, we have also confirmed a likely anatomical candidate for the effects observed $\alpha 4$ -KO mice.

Our virus placements are centred in the NAc Core based on the efficacy of this manipulation in previous studies on cocaine-related behaviour (Macpherson, 2013). This is in contrast with those experiments which only found a similar effect when virus was infused into the NAc Shell but not Core of rats (Rewal *et al.*, 2012). As we used mice in our experiments these sub-regions may be closer, where they are more distinct in the larger rat brain, and therefore both receive some virus. Although the rat and mouse brains are anatomically highly similar, many major functional and behavioural differences relating to addiction have been described (reviewed, Ellenbroek and Yoon, 2016).

Unfortunately using DID experiments we are unable to examine the motivational/appetitive changes underlying this behaviour. Food remained available *ad libitum* during drinking sessions, so it is unlikely that we are observing differences in calorie-seeking behaviour and we did not observe differences in water consumption. We therefore provide good evidence that differences between genotypes are specific to alcohol consumption.

We suggest two opposing hypotheses; increased tonic inhibition mediated by $\alpha 4$ -GABA_ARs either reduces the "reward" value of ethanol or it enhances satiation such that mice are sated after drinking less alcohol. Time-course data for drinking is not available from our experiment but if gathered in future experiments may indicate whether $\alpha 4$ -KO mice satiate faster than $\alpha 4$ -WTs. As reviewed earlier, downregulation of $\alpha 4$ or δ reduced operant ethanol self-administration (Rewal *et al.*, 2012) however there was no difference between genotypes in the first 5 minutes of drinking sessions, following several reinforcers, which indicates that this is a difference in consummatory rather than appetitive behaviour. Conversely mice carrying a mutation of the $\beta 1$ subunit

which potentiates GABA_A receptor activity maintained high rates of responding throughout sessions whilst wild-types slowed their rate of as the session progressed consistent with them satiating on alcohol (Anstee *et al.*, 2013).

Given the upregulation of $\alpha 4$ following chronic ethanol administration (Liang *et al.*, 2007) we may consider whether this represents a maladaptive or homeostatic response to ethanol. We found that reduction in $\alpha 4$ -GABA_AR activity opposes drinking suggesting upregulation of $\alpha 4$ is likely a maladaptive change. This may however be opposed by reduced synaptic GABA transmission in MSNs (Wilcox *et al.*, 2014) acting as a homeostatic mechanism.

It is necessary to establish whether this control of drinking by $\alpha 4$ receptors in the NAc is bidirectional if they are to be considered as a therapeutic target. Previous results using GABA_A agonists would predict this (Boyle *et al.*, 1993; Rassnick *et al.*, 1993; Hyytiä and Koob, 1995). We sought to use $\alpha 4$ -KO mice in similar experiments, as a negative control, so we can more reliably attribute the behavioural effects of GABA_A agonists to $\alpha 4$ -GABA_ARs (i.e. where agonists are effective in $\alpha 4$ -WT but not $\alpha 4$ -KO animals).

Contrary to studies in rats (Boyle *et al.*, 1993) we did not find any evidence that systemic administration of THIP specifically reduced ethanol consumption. We found a genotype specific decrease in consumption of both ethanol and water following a high dose (16mg/kg IP) of THIP therefore we hypothesise that it is due to a sedative effect of IP THIP at higher doses in mice. Notably, $\alpha 4$ -KO mice, which did not show the THIP induced reduction in water consumption, are resistant to some of the motor incoordinating effects of THIP (Chandra *et al.*, 2006) and in previous studies in our lab we have observed that THIP dose-dependently decreases locomotor activity in $\alpha 4$ -WT but not $\alpha 4$ -KO mice (Macpherson, 2013).

Our data suggests that the reduced ethanol consumption was due to THIPs sedative effects rather than acting on receptors in the NAc which are likely to mediate the reinforcing properties of alcohol (Rewal *et al.*, 2009). This replicates another study that reported IP THIP decreased both water and ethanol consumption in C57BL/6J mice in a DID test (Moore *et al.*, 2007). Ramaker and colleagues (2012) examined the effect of IP THIP on C57BL/6J mice using a

limited-access 'two-bottle choice' test instead and found reductions in ethanol consumption, but not water, after 8mg/kg or 16mg/kg doses and thus concluded that THIP reduced ethanol preference. Strangely this indicates dissociated effects of THIP on ethanol consumption in 'two-bottle-choice' and DID. It is possible that THIP reduced ethanol but not water consumption at those doses because of compounding sedative effects of both THIP and ethanol itself. The authors reported that they observed sedative effects following the 16mg/kg dose.

There are two problems with systemic dosing of THIP: a lack of anatomical resolution and the resultant off-target sedative effects. Since $\alpha 4$ downregulation in the NAc was sufficient to reduce ethanol consumption we attempted to demonstrate bidirectional control by $\alpha 4$ -GABA_ARs in this region. Previously we have observed opposite effects of targeted intracranial THIP infusions compared to systemic THIP injection. Infusion of THIP directly to the NAc of WT C57BL/6J mice increased ethanol but not water consumption in DID (Macpherson, Stephens and King, unpublished data). By repeating this DID experiment in $\alpha 4$ -KO animals we hoped to demonstrate that this effect was specifically mediated by $\alpha 4$ -GABA_ARs and not THIP acting non-specifically at other receptors in the NAc.

If deletion of $\alpha 4$ -GABA_ARS abolished the ability of THIP to increase drinking we could conclude that $\alpha 4$ -GABA_ARs in the NAc bidirectionally modulate ethanol consumption. Our current study failed to replicate an intra-accumbal THIP induced increase in drinking in $\alpha 4$ -WT mice, and thus a null effect in the $\alpha 4$ -KO mice could not be interpreted. Notably we observed a trend towards THIP increased drinking (g/kg) in $\alpha 4$ -WT (mean = 1.13, SEM = 0.013) but not $\alpha 4$ -KO mice (mean = 0.2595, SEM = 0.0207, $p=0.131$, Observed Power = 0.312). To further examine the role of $\alpha 4$ -GABA_ARs we also used infusions of the GABA_A agonist DS2 as it is also highly selective for $\alpha 4\beta\delta$ receptors (Wafford *et al.*, 2009). Again, we did not demonstrate a significant increase in ethanol consumption following intra-NAc DS2 although we observed a trend towards increased ethanol consumption in $\alpha 4$ -WT (mean = 1.0507, SEM = 0.046) but not $\alpha 4$ -KO mice (mean = 0.2886, SEM = 0.021, $p=0.158$, Observed Power = 0.209).

It is notable that both $\alpha 4\beta\delta$ receptors agonist treatments trended towards increased ethanol consumption in $\alpha 4$ -WT but not $\alpha 4$ -KO animals.

Together these results conflict with a previous study by Ramaker and colleagues (2015) in which intra-NAc THIP reduced ethanol consumption in a limited access 'Two Bottle Choice' test. Again, we find a dissociation of 'two-bottle-choice' and DID whereby we observe the opposite effect of this manipulation on drinking (Macpherson, Stephens & King, unpublished data). Other notable experimental differences were a lower dose of THIP (200mg vs ~ 265 mg), and infusion into the NAc-Shell rather than centred in the Core as in our experiments.

Concentrations of THIP were chosen based on a previous study testing the effect of NAc core infusions of THIP in $\alpha 4$ knockout and wildtype mice (Maguire *et al.*, 2014). THIP is most selective for $\alpha 4\beta\delta$ receptors at between 1 and $10\mu\text{M}$ whereby it increases current by ~ 50 - 100% only at $\alpha 4\beta\delta$ receptors but not at other GABA_AR isoforms (Mortensen *et al.*, 2010). However, at higher doses of $100\mu\text{M}$ to 1mM it acts as a 'superagonist' increasing $\alpha 4\beta\delta$ current by 250% but also increasing current by 100% at other GABA_AR isoforms (Mortensen *et al.*, 2010). We used high dose of THIP ($0.5\mu\text{l}$ of 3mM) because this 'superagonist' concentration it has been behaviourally active in previous experiments on cocaine conditioned behaviour (Maguire *et al.*, 2014; Macpherson *et al.*, 2016) and we can control for the off-target effects by comparing $\alpha 4$ -WT and $\alpha 4$ -KO mice.

It is unexpected that intra-NAc THIP in the shell would reduce drinking since downregulation of $\alpha 4\beta\delta$ receptors in the same region also reduced drinking. If $\alpha 4$ -KOs are satiating faster due to less tonic inhibition (as suggested above) then THIP mediated inhibition would likely be opposing the 'satiating' effects of alcohol which may explain this result. To explain our conflicting finding we also suggest that infusion to the NAc-Core rather than NAc-Shell may have different effects.

Studies in which the Core or Shell were pharmacologically inactivated prior to ethanol-rewarded PIT experiments revealed distinct roles for the sub-regions in ethanol reinforcement (Corbit, Fischbach and Janak, 2016). They reported that the NAc core was required for responding to ethanol-predictive cues whilst the

shell mediated the influence of environmental context. Since DID is a home-cage test environmental context is controlled any stimuli related to the presentation of alcohol (e.g. smell & taste) may be used as discrete cues which are influenced by $\alpha 4$ -GABA_AR activity in the NAc Core. Thus $\alpha 4$ -GABA_AR agonism there may reduce the reinforcing properties of ethanol-related sensory stimuli during consumption resulting in faster satiation.

Previous studies have suggested that D1 and D2 MSN populations may have distinct roles in ethanol drinking. D1 antagonists/agonists in the NAc-shell but not core reduce/enhance operant responding for ethanol (Hauser *et al.*, 2015) indicating that D1 activity enhances motivation for ethanol. These populations also display distinct neuroadaptations following binge-like drinking. Two weeks of DID sessions shifted D1 MSNs from a state of LTD to LTP and D2 MSNs from LTP to LTD (Ji *et al.*, 2017).

We therefore attempted to examine whether tonic inhibition by $\alpha 4$ -GABA_ARs on D1 or D2 MSN populations specifically may mediate distinct effects on binge-like alcohol consumption. No effect on DID ethanol consumption was apparent in $\alpha 4$ -D1-KO or $\alpha 4$ -D2-KO specific knock-outs. Thus, it appears $\alpha 4$ -GABA_AR activity is required in both D1 and D2 pathways to mediate ethanol DID. This differentiates these effects from the dissociable roles of $\alpha 4$ on D1 and D2 MSNs in cocaine CPP (Maguire *et al.*, 2014) or conditioned-reinforcement respectively (Macpherson *et al.*, 2016). It is possible that D1 MSN activity underlies motivational behaviour (Maguire *et al.*, 2014; Hauser *et al.*, 2015) whilst D2 MSN activity processes ethanol-predictive cues such as smell or taste (Macpherson *et al.*, 2016; Owesson-White *et al.*, 2016). In this case normal tonic inhibition in either population may compensate for disinhibition in the other.

At present our results do not suggest $\alpha 4$ modulation of total ethanol consumption is D1 or D2 dependant. Systemic pharmacological manipulations of D2 receptors have resulted in changes to the time-course of drinking by reducing initial drinking earlier in sessions but increasing it later in sessions (Spoelder *et al.*, 2016). We may therefore expect similar patterns in $\alpha 4$ -D2-KO mice. Our experimental set-up was not equipped to detect differences in patterns of drinking within the DID trial period which might be present in $\alpha 4$ -D1-KO/ $\alpha 4$ -D2-KO mice. Further experiments using more sensitive measurements of

fluid consumption over time (e.g. using lickometers) could indicate at what rate mice drink and satiate over the course of each session. Similarly, operant self-administration experiments could be used to investigate time-course of ethanol consumption or appetite based on rates of responding over time in a similar delayed then increased pattern.

Time-course data may also indicate whether reduced consumption in $\alpha 4$ -KOs is a result of faster satiation. This would be indicated if levels of drinking are similar early in the session but more rapidly decline. Notably operant ethanol self-administration in rats following intra-NAc RNAi knockdown of $\alpha 4$ was similar to controls in the first 5 minutes of sessions but reduced overall (Rewal *et al.*, 2012). Operant self-administration may also be a useful model to isolate $\alpha 4$ -GABA_ARs role in motivational aspects of ethanol consumption rather than simply measuring consummatory behaviour. Our AAV-Cre viral method could be used to knockdown $\alpha 4$ -GABA_ARs prior to similar operant self-administration studies in mice.

Since $\alpha 4$ -GABA_ARs in the NAc appear to be a significant modulator of ethanol consumption it may be of interest to investigate other regions in which $\alpha 4$ is expressed using similar methods. The thalamus is a good candidate as it is where $\alpha 4$ is most highly expressed (Sur *et al.*, 1999b; Schwarzer *et al.*, 2001) and ethanol is known to potentiate GABAergic tonic inhibition there in wild-type but not $\alpha 4$ -KO mice (Jia, Pignataro and Harrison, 2007; Jia *et al.*, 2008). If depression of the thalamus is necessary to promote ethanol consumption $\alpha 4$ knockdown may result in reduced drinking.

Additionally, the Dorsomedial Striatum (DMS) has been implicated in maladaptive increase of GABA transmission, as measured by slice electrophysiology in the presence of GABA, in D2 MSNs following chronic DID (Cheng *et al.*, 2017). If this is disrupted by $\alpha 4$ knockdown this would implicate extrasynaptic GABA_ARs in mediating such changes and therefore as a therapeutic target for alcoholism.

Chapter 4

The role of $\alpha 4$ -containing GABA_A receptors in locomotor behaviour and instrumental responding and their potentiation by cocaine

4.1. Introduction

4.1.1. Locomotor behaviour

Locomotor activity, defined as movement from one location to another, is a crucial component of all animals' behaviour. It underlies their ability to explore the environment which is critical for approaching salient stimuli, such as food or sex, and avoiding aversive stimuli, such as predators. Animals display an initial increased level of exploratory locomotor activity when placed in a novel environment or in response to novel stimuli however this activity rapidly decreases if the salience is determined to be neutral (Harris, 1943). To a lesser extent some spontaneous locomotor activity occurs in habituated environments, suggesting that animals also display a basal level of locomotor activity (Robbins, 1977; Paulus and Geyer, 1993).

It is well established that locomotor activity is enhanced by drugs that facilitate transmission at dopamine synapses, moreover it is reduced by dopamine antagonists or lesions of dopaminergic systems (Kelly, Seviour and Iversen, 1975; Kelly and Iversen, 1976; Wachtel and Anden, 1978; Fray *et al.*, 1980). Psychostimulant drugs, which potentiate dopamine transmission, dose-dependently modulate locomotor activity in an inverted U-shaped function (Isaacson, 1978). Acute administration of psychostimulants potentiates locomotor activity until at high doses their effects become so intense as to disrupt organised locomotor behaviour and therefore induces severe behavioural stereotypy (Randrup and Munkvad, 1967; Bhattacharyya and Pradhan, 1979).

When dopamine agonists were infused directly into various regions of the rat forebrain the behavioural outcomes suggested that locomotor stimulation was primarily mediated by the NAc, and stereotyped behaviours from the dorsal striatum (Costall *et al.*, 1977; Campbell *et al.*, 1997). Locomotor behaviour resulting from dopamine activity in the NAc is notable in the study of addiction

as all drugs of abuse increase dopamine release in the NAc (Wise, 1987, 1988; Di Chiara and Imperato, 1988; Pettit and Justice, 1989). Repeated administration of psychostimulants results in increased dopamine release in the NAc and increased locomotor activity (Di Chiara and Imperato, 1988; Kalivas and Duffy, 1990; Kalivas and Stewart, 1991).

The predominant neuronal type within the NAc are GABAergic MSNs (Kemp and Powell, 1971) therefore it is likely that GABAergic transmission is involved in controlling baseline and psychostimulant-potentiated locomotor activity. Systemic injection of GABA agonists decreased locomotor activity although this appeared to be primarily via GABA_B receptors (Agmo and Giordano, 1985). Systemic administration of drugs blocking GABA breakdown attenuated baseline and amphetamine-potentiated locomotor activity (Grimm *et al.*, 1975; Cott and Engel, 1977). Direct injections of GABA into the NAc affected a bimodal response in locomotion, with low doses inducing a small increase, and larger doses producing a reduction (Wachtel and Anden, 1978; Jones, Mogenson and Wu, 1981) indicating multiple, possibly conflicting, roles for GABA in mediating locomotor activity..

Activation of GABA_A receptors in the NAc appears to oppose locomotor activity and its potentiation by psychostimulants. Systemic administration of a GABA-transaminase inhibitor, ethanolamine-O-sulphate, abolished the ability of intra-NAc dopamine injections to potentiate locomotor activity, but has no significant effect on baseline locomotor activity (Pycock and Horton, 1976). Injection of the GABA_A receptor *agonist* Muscimol in the NAc core reduced dopamine receptor-mediated locomotor behaviour in mice (Akiyama *et al.*, 2003; Akiyama *et al.*, 2004) whilst intra-NAc microinjections of the GABA_AR *antagonist* picrotoxin enhanced baseline and intra-NAc amphetamine-potentiated locomotor responses (Pycock and Horton, 1976; Jones, Mogenson and Wu, 1981; Wong *et al.*, 1991).

There is also recent evidence that extrasynaptically located GABA_ARs specifically play a functional role in mediating locomotor activity. Systemic administration of THIP, a GABA_A antagonist selective for $\alpha 4\beta\delta$ receptors, reduces baseline locomotor activity and attenuates enhanced locomotion following intra-NAc administration of the glutamate agonist 6,7-ADTN (Arnt, 1981; Agmo and Giordano, 1985; Herd *et al.*, 2009; Vashchinkina *et al.*, 2012).

Previously we have reported that constitutive deletion of the $\alpha 4$ subunit does not affect baseline or cocaine-induced locomotor activity. In wild-type mice intra-NAc THIP reduced locomotor activity and attenuated cocaine-induced locomotor activity however these effects were abolished in $\alpha 4$ -KO mice (Macpherson, 2013).

Manipulations of both D1 and D2 type striatal MSNs alter locomotor activity although effects vary depending on the sub-region. Systemic administration of D1 agonists increases locomotion (Schindler and Carmona, 2002). Evidence for the effects of D2 receptor agonists is mixed with some studies finding it potentiated and others that it attenuated locomotion depending on dose and timing (Schindler and Carmona, 2002; Stuchlik *et al.*, 2007). Optogenetic stimulation of D1 or D2 MSNs in the dorsomedial striatum resulted in increased or decreased locomotion respectively (A. V Kravitz *et al.*, 2010).

Within the NAc D1- and D2- expressing neurons mediate similar effects on locomotor activity and its potentiation by cocaine although D1-expressing neurons are predominantly involved (Bruhwyler *et al.*, 1991; Mazurski and Beninger, 1991). Injections of D1 agonists into the NAc of rats greatly increased locomotor activity whilst D2 agonists did so more modestly and when co-administered their effects were additive (Dreher and Jackson, 1989; Gong *et al.*, 1999). Intra-NAc D1 and D2 antagonists attenuated cocaine-induced locomotor activity (Kita *et al.*, 1999) whilst D1 agonists in the NAc Shell enhanced cocaine-induced locomotion as did D2 agonists although to a lesser extent (Bachtell *et al.*, 2005a). Similarly, targeted blockade of neurotransmission by a tetanus toxin in either Direct or Indirect pathway neurons within the NAc abolished locomotor potentiation by cocaine or methamphetamine in mice (Hikida *et al.*, 2010).

We therefore investigated the role of $\alpha 4$ containing GABA_ARs receptors, specifically on D1 and D2 neurons, in locomotor behaviour. Here we use $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO conditional knock-out mice (see chapter 2) in a cocaine dose-response experiment where locomotor activity was recorded following various doses of cocaine.

4.1.2. Progressive Ratio

It has been suggested that potentiated locomotor responses following intra-NAc infusions of dopamine agonists may result from a general facilitation of approach-investigation behaviour which is subsequently directed by environmental conditions (Ikemoto and Panksepp, 1999). In a standard locomotor activity chamber, lacking interactive stimuli, increased NAc dopamine transmission may simply stimulate exploratory behaviour including locomotion and rearing. Alternatively, in other situations this general stimulation may facilitate other approach behaviours such as instrumental responding or conditioned activity in operant tasks (Taylor and Robbins, 1986; Cador, Taylor and Robbins, 1991; Kelley and Delfs, 1991).

In instrumental responding experiments animals are required to perform a behaviour, such as lever-pressing, to attain a 'reinforcer' such as food or drugs. Schedules of reinforcement describe the number of responses required, for example in a Fixed Ratio schedule a specified number of responses is rewarded (e.g. FR4 = 4 lever-presses) (Ferster, 1957). Under a Progressive Ratio schedule the number of instrumental responses required is increased by a fixed increment each time a reinforcer is attained until the animal stops responding, i.e. reaches its 'breakpoint' (Hodos, 1961). This measure has been used to investigate motivation for natural rewards (Hodos, 1961) and drugs of abuse in self-administration experiments including psychostimulants (Griffiths *et al.*, 1975), alcohol (Ritz *et al.*, 1994; Brown, Jackson and Stephens, 1998) and opiates (Hoffmeister, 1979; Roberts and Bennett, 1993).

As with locomotor activity it has been observed that dopamine manipulations in the NAc are sufficient to affect instrumental responding and breakpoints during PR schedules. Lesion of dopaminergic cells in the NAc by 6-OHDA significantly reduced responding during high-increment, but not low-increment, PR schedules (Salamone *et al.*, 1999, 2001). Intra-NAc Shell injections of amphetamine significantly enhanced the number of active lever responses during a food reinforced PR schedule (Zhang, Balmadrid and Kelley, 2003).

Importantly, dopamine depletions or antagonism in the NAc do not impair appetite for food or disrupt of primary food motivation (Ungerstedt, 1971; Koob

et al., 1978; Bakshi and Kelley, 1991; Salamone *et al.*, 1993). In dopamine deficient transgenic mice, restoration of dopamine production in caudate putamen, but not NAc, was sufficient to rescue feeding behaviour (Szczycka *et al.*, 2001). It has therefore been suggested that NAc dopamine transmission increases instrumental responding by increasing the level of work that animals are willing to do for similar reinforcement (Salamone *et al.*, 1999).

Systemic and intra-NAc administration of psychostimulants, including cocaine, increases responding and breakpoints for food under progressive ratio schedules (Poncelet *et al.*, 1983; Zhang, Balmadrid and Kelley, 2003). Similarly, cocaine increased breakpoints for self-administration of ethanol (Brown and Stephens, 2002), cocaine (Roberts and Bennett, 1993) and opiates (Duvauchelle, Sapoznika and Kornetskya, 1998). It has therefore been cautioned that high breakpoints for psychostimulants relative to other drugs may be due to their stimulant effects rather than greater efficacy as primary reinforcers (Jones *et al.*, 1995; Brown and Stephens, 2002). The hypothesis that psychostimulants increase the efficacy of primary reinforcers cannot be discounted however, in the case of food, it appears unlikely since at the same dose cocaine both increases PR breakpoints whilst reducing consumption and preference for sucrose (Balopole, Hansult and Dorph, 1979; Brown and Stephens, 2002).

Again, GABAergic activity within MSNs is likely to modulate these behaviours. Specifically agonism of GABA_A receptors in the NAc shell, and to a lesser extent NAc Core, directly promotes food consumption (Stratford and Kelley, 1997; Basso and Kelley, 1999) as well as instrumental responding for food under progressive ratio schedules (Wirtshafter and Stratford, 2010). To date no published studies have investigated whether GABAergic activity in the NAc modulates the ability of cocaine to facilitate instrumental responding for natural rewards under progressive ratio schedules. Previously we found no difference in instrumental responding for primary rewards under fixed or progressive ratio schedules in $\alpha 4$ -KO mice (see chapter 2) either at baseline or following cocaine administration (Macpherson, 2013).

Systemic or intra-NAc administration of *either* D1 or D2 receptor antagonists reduces the breakpoint of responding for food in progressively escalating schedules of reinforcement (Hubner and Moreton, 1991; Aberman, Ward and

Salamone, 1998; Barbano, Le Saux and Cador, 2009). Similarly optogenetic stimulation of *either* D1 or D2 MSNs in the NAc increases Progressive Ratio (PR) responses and breakpoints for food (Soares-Cunha *et al.*, 2016). Systemic and intra-NAc D1 and D2 antagonists reduced breakpoints in a PR schedule of cocaine self-administration (Hubner and Moreton, 1991; Bari and Pierce, 2005).

Previously we have reported that constitutive deletion of the $\alpha 4$ subunit or virally mediated RNAi knockdown of $\alpha 4$ subunits in the NAc are sufficient to increase rates of instrumental responding for reward-paired stimuli in a test of conditioned reinforcement (CRf). In addition we found a similar effect when the $\alpha 4$ -GABA_ARs was conditionally deleted in D2 but not D1 MSNs (Macpherson *et al.*, 2016). Further experiments on instrumental responding for primary reinforcers could determine whether these effects are general to approach-investigation behaviour or specific to cue-driven responses. Additionally, given that GABA_A receptor activity in the NAc appears to mediate primary reinforcement of food (Wirtshafter and Stratford, 2010), we wished to investigate whether this might underlie $\alpha 4$ -GABA_AR mediated effects on CRf (Macpherson *et al.*, 2016). Comparing CRf experiments with progressive ratio schedules may allow us to dissociate effects resulting from increased efficacy of the primary reinforcer vs increased response to secondary-reinforcers.

Despite their widespread use Progressive Ratio tests (PR) are subject to problems of interpretation. Increased breakpoint values may indicate increased motivation however perseveration may also result from impaired learning flexibility or facilitation of general locomotor output, as discussed above (Ikemoto and Panksepp, 1999; Brown and Stephens, 2002). Comparing similar manipulations in PR, locomotor and CRf experiments may also indicate whether differences in CRf responding result from such generalised behaviour.

We therefore investigated the role of $\alpha 4$ containing GABA_ARs receptors, specifically on D1 and D2 neurons, in fixed and progressive ratio schedules of reinforcement by sucrose. Here we use $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO conditional knock-out mice (see chapter 2) in a cocaine dose-response experiment where instrumental responding and PR breakpoints were recorded at baseline and following various doses of cocaine.

4.2. Methods

4.2.1. Animals

Conditional dopamine D1/D2 expressing neuron specific $\alpha 4$ -subunit knockout mice were created by crossing "Floxed" $\alpha 4$ -subunit homozygous transgenic mice (strain name; B6.129-Gabra4tm1.2Geh/J, supplied by The Jackson Laboratory, ME, USA) with either dopamine receptor D1 or D2 neuron specific Cre-recombinase hemizygous transgenic mice (strain name; $\alpha 4$ -D1-KO = B6.FVB(Cg)-Tg(Drd1a-cre)EY217Gsat/Mmucd, $\alpha 4$ -D2-KO = B6.FVB(Cg)-Tg(Drd2-cre)ER44Gsat/Mmucd, supplied by Mutant Mouse Regional Resource Centers (MMRRC), ME, USA). Breeding was conducted as described in Figure 1.2.

Male and female GABA_AR $\alpha 4$ D1- or D2-expressing neuron specific $\alpha 4$ wildtype ($\alpha 4$ -D1-WT/ $\alpha 4$ -D2-WT) and knockout ($\alpha 4$ -D1-KO/ $\alpha 4$ -D2-KO) mice on a C57Bl/6J background strain, weighing between 20-30g, were housed in groups of 2-3, or separately for surgery animals, with food and water available ad libitum. A 12hr light/dark cycle was used (lights on at 7:00 A.M.) with holding room temperature maintained at 21 \pm 2°C and humidity 50 \pm 5%. All injections, infusions and behavioural testing were performed between 2:00 P.M. and 5:00 P.M. All procedures were conducted in accordance to Animals (Scientific Procedures) Act 1986, following ethical review by the University of Sussex Ethical Review Committee.

4.2.2. Drugs

Cocaine Hydrochloride was obtained from Macfarlan Smith (Edinburgh, UK). Cocaine was dissolved to desired concentrations in 0.9% saline, and administered IP at an injection volume of 10 ml/kg.

4.2.3.1 Locomotor Activity

We recorded locomotor activity in 16 annular black Perspex runways, (diameter 24cm, annula width 6.5cm), placed atop a clouded Perspex sheet on an elevated frame. A digital camera positioned beneath the sheet captured the silhouettes of the boxes' edges and the mice within them, which was then relayed to a computer to be recorded. A MatLab (MathWorks, Cambridge, UK) video analysis

programme and Excel macro converted the video data into a measure of the distance travelled in metres.

The locomotor response to acute cocaine at various doses was tested in $\alpha 4$ -D1-WT, $\alpha 4$ -D1-KO, $\alpha 4$ -D2-WT and $\alpha 4$ -D2-KO mice (n=8 per genotype). Prior to testing there were two habituation days, on the first day mice were habituated to the equipment for 60 minutes, on the second day mice received an *i.p.* injection of saline prior to being placed in the apparatus. All animals underwent five test days in a within-subjects, Latin square design, during which they were administered saline, 3, 10, 20, 30mg/kg prior to being placed in the apparatus.

4.2.3.2. Progressive ratio test

Instrumental responding was measured using 8 operant chambers (Med Associates Inc, Vermont, USA), each housed within a light-resistant, sound-attenuating cubicle. The front wall was fitted with a liquid dipper, located between 2 ultrasensitive mouse levers. Head entries into the liquid dipper magazine were detected using an infrared beam. Each operant chamber possessed a single house light located on the wall opposite the levers. In all sessions both levers were presented but only one 'active lever' resulted in reinforcement, this was alternated between left and right to avoid side-bias. Liquid dippers presented 0.2ml of 10% sucrose solution as reinforcers.

Mice were food deprived to 90% of free-feeding bodyweight and trained to press the active lever for 10% sucrose solution in operant conditioning chambers. On day 1 mice underwent a 15h training session which included the dark phase and during which 10% sucrose solution was available on an FR1 schedule.

Fixed ratio – Mice were tested in daily one-hour sessions (between 9:00 A.M. and 1:00 P.M.) during which the FR was 1, 2 or 4 for three consecutive sessions each.

Progressive ratio – Mice were then tested in one session using a high-increment PR schedule in which the FR was doubled each time a reinforcer was attained (i.e. 1, 2, 4, 8, 16... etc.). On the following two days mice were tested in sessions where saline or cocaine (10mg/kg) was administered directly prior to being placed in operant chambers in a within-subjects, counterbalanced design with each mouse receiving both treatments.

Using the same PR schedule all mice underwent five test daily sessions before which they were administered saline, 3, 10 or 30mg/kg directly prior to being placed in operant chambers in a within-subjects, Latin-square design with each mouse receiving all doses.

4.2.4. Statistics

All analyses were initially carried out including sex as a between-subjects independent variable however we found no main effects or interactions with other variables and sex was therefore excluded in secondary analysis for clarity.

4.2.4.1. Locomotor activity during Cocaine Dose Response

Baseline locomotor activity was assessed by a multivariate ANOVA using genotype as the between subjects factor and locomotor activity during habituation sessions and initial I.P. saline session as the dependant variables.

Locomotor activity data for the cocaine dose response study was assessed by a repeated-measures ANOVA using genotype as the between-subjects factor, cocaine dose as a within-subjects factor and locomotor activity as the dependent variable.

4.2.4.2. Instrumental responding under Fixed Ratio schedules

Accuracy of instrumental responding was assessed using a multi-factor repeated measures ANOVA using FR and lever as within subjects factors, genotype as between subjects factors and lever-presses as the dependent variable.

Reinforced instrumental responding was assessed by an ANOVA using genotype as a between-subjects factor and reinforcers earned as the dependant variable.

4.2.4.3. Instrumental responding under Progressive Ratio schedules

PR instrumental responding data was assessed by a multivariate ANOVA using genotype as a between-subjects factor and PR breakpoint, active lever presses, and inactive lever presses as the dependant variables.

4.2.4.4. Cocaine (10mg/kg) Potentiation of Progressive Ratio

PR instrumental responding data for the cocaine dose response study was assessed by a repeated-measures ANOVA using genotype as a between-subjects

factor, drug treatment as the within-subjects factor and PR breakpoint, active lever presses and inactive lever presses as the dependant variables.

4.2.4.5. Progressive Ratio Cocaine Dose Response

Instrumental responding data for the cocaine dose response study was assessed by a repeated-measures ANOVA using genotype as a between-subjects factor, cocaine dose as the within-subjects factor and PR breakpoint, active lever presses and inactive lever presses as the dependant variables.

4.3. Results

4.3.1. Locomotor activity cocaine-dose-response in $\alpha 4$ -D1-WT/ $\alpha 4$ -D1-KO mice

Baseline locomotor activity during habituation was similar in both $\alpha 4$ -D1-WT and $\alpha 4$ -D1-KO mice (Figure 4.1; non-significant effect of genotype, $F_{(1,14)} = 2.172$, $p = 0.163$; NS) and baseline locomotor activity following I.P. saline administration was similar in both genotypes (non-significant effect of genotype, $F_{(1,14)} = 1.549$, $p = 0.234$; NS).

Acute administration of cocaine dose-dependently increased locomotor activity in both $\alpha 4$ -D1-WT and $\alpha 4$ -D1-KO mice (Figure 4.1; significant main effect of dose, $F_{(4,56)} = 36.57$, $p < 0.001$). We observed an increased response to cocaine in $\alpha 4$ -D1-KO mice relative to $\alpha 4$ -D1-WT littermates (significant main effect of genotype, $F_{(1,14)} = 6.61$, $p < 0.05$; non-significant dose by genotype interaction, $F_{(4,56)} = 1.16$, $p = 0.34$; NS).

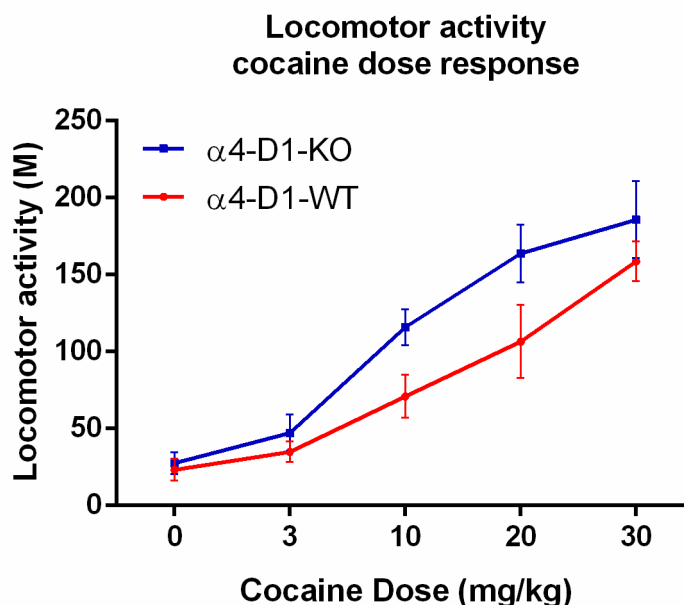


Figure 4.1. Effect of acute intraperitoneal cocaine on distance travelled over 60 minutes in α4-D1-WT and α4-D1-KO mice. Cocaine dose-dependently increased locomotor activity in both α4-D1-WT (n=8) and, to a greater extent, in α4-D1-KO mice (n=8) mice ($p < 0.05$). Error bars represent SEM.

Baseline locomotor activity was significantly greater in the first 15 minutes of the session (Figure 4.2; significant main effect of time-bin, $F_{(11,65)} = 92.73$, $p < 0.001$) and cocaine potentiation of locomotor activity occurred in the first 15 minute of the session (Figure 4.2; significant time-bin by dose interaction $F_{(11,65)} = 6.48$, $p < 0.001$). We did not observe a difference in the timecourse of locomotor behaviour between genotypes (Figure 4.2; non-significant time-bin by genotype interaction $F_{(11,65)} = 0.705$, $p = 0.73$, NS).

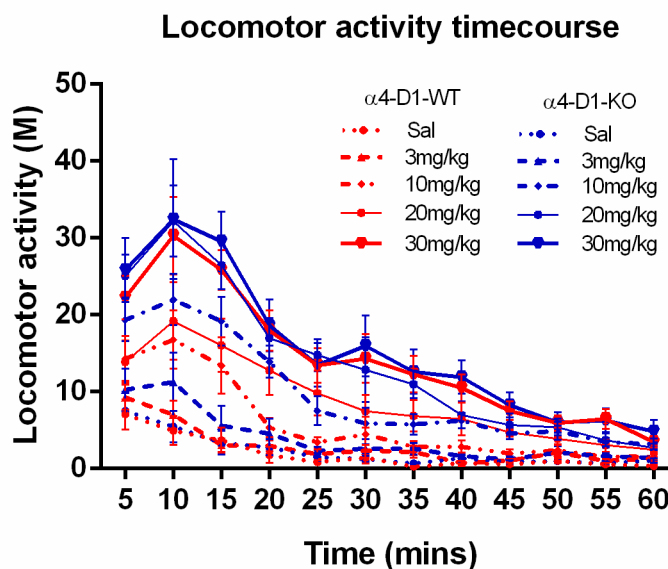


Figure 4.2. Timecourse of activity over 60 minutes following I.P. injections of saline and cocaine at 3, 10, 20 or 30 mg/kg to $\alpha 4$ -D1-WT (n=8) and $\alpha 4$ -D1-KO (n=8) mice. Error bars represent SEM.

4.3.2. Locomotor activity cocaine-dose-response in $\alpha 4$ -D2-WT/ $\alpha 4$ -D2-KO mice

Baseline locomotor activity during habituation was similar in both genotypes (Figure 4.3; non-significant effect of genotype, $F_{(1,14)} = 1.564$, $p = 0.232$; NS) and baseline locomotor activity following I.P. saline administration was similar in both genotypes (non-significant effect of genotype, $F_{(1,14)} = 1.7$, $p = 0.213$; NS).

Acute administration of cocaine dose-dependently increased locomotor activity equally in $\alpha 4$ -D2-WT and $\alpha 4$ -D2-KO mice (Figure 4.3; significant main effect of dose, $F_{(4,56)} = 72.09$, $p < 0.001$; non-significant main effect of genotype, $F_{(1,14)} = 0.001$, $p = 0.98$ NS; non-significant dose by genotype interaction, $F_{(4,56)} = 0.87$, $p = 0.461$, NS).

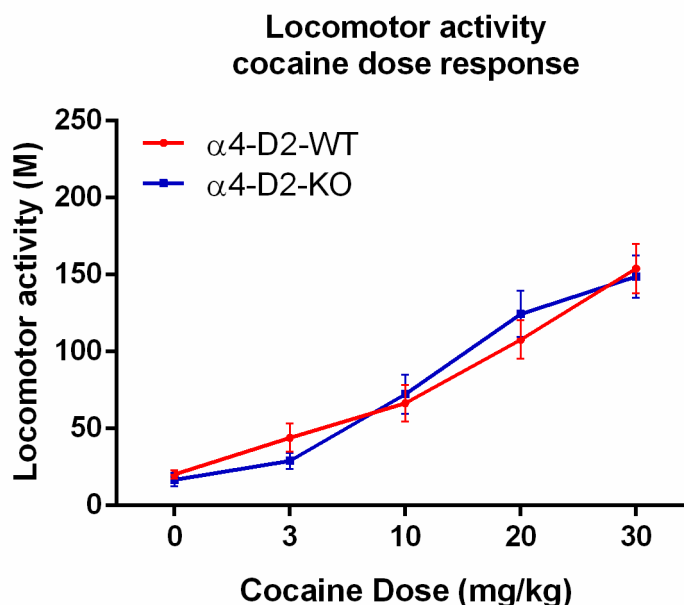


Figure 4.3. Effect of acute intraperitoneal cocaine on distance travelled over 60 minutes in α4-D2-WT and α4-D2-KO mice. Cocaine dose-dependently increased locomotor activity equally in both α4-D2-WT (n=8) and α4-D2-KO mice (n=8) mice. Error bars represent SEM.

Baseline locomotor activity was significantly greater in the first 15 minutes of the session (Figure 4.4; significant main effect of time-bin, $F_{(11,65)} = 109.87$, $p < 0.001$) and cocaine potentiation of locomotor activity occurred in the first 15 minute of the session (Figure 4.4; significant time-bin by dose interaction $F_{(11,65)} = 12.997$, $p < 0.001$). We did not observe a difference in the timecourse of locomotor behaviour between genotypes (Figure 4.4; non-significant time-bin by genotype interaction $F_{(11,65)} = 0.326$, $p = 0.61$, NS).

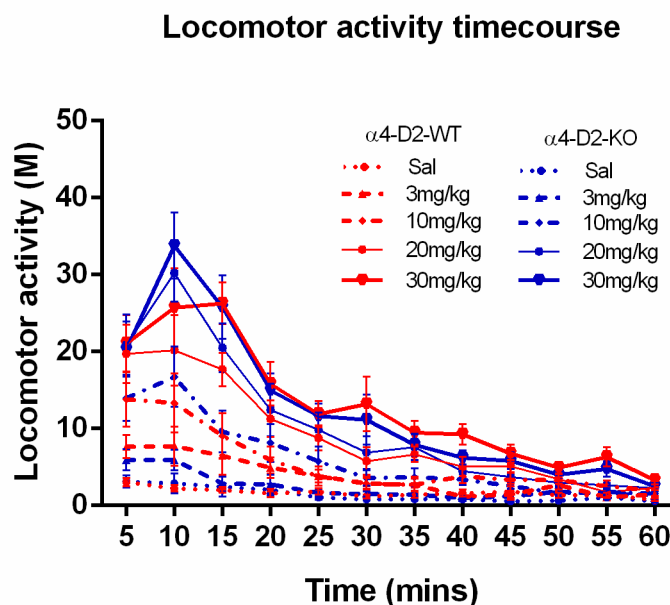


Figure 4.4. Time-course of activity over 60 minutes following I.P. injections of saline and cocaine at 3, 10, 20 or 30 mg/kg to α4-D2-WT (n=8) and α4-D2-KO mice (n=8). Error bars represent SEM.

4.3.3. Instrumental responding under Fixed Ratio schedules in α4-D1-WT/α4-D1-KO mice

Mice responded significantly higher on the active lever than inactive lever and this was similar in both genotypes (Table 1.1; significant effect of lever, $F_{(2,12)} = 53.77$, $p < 0.001$; non-significant effect of genotype, $F_{(2,12)} = 0.376$, $p = 0.55$; non-significant genotype by lever interaction, $F_{(2,12)} = 0.417$, $p = 0.53$, NS).

Higher FR requirements elicited more active lever presses, but not inactive lever presses, from both genotypes (Figure 4.5; significant effect of FR, $F_{(2,12)} = 24.49$, $p < 0.001$; non-significant FR by genotype interaction, $F_{(2,12)} = 0.378$, $p = 0.69$, NS; significant FR by lever interaction $F_{(2,12)} = 24.59$, $p < 0.001$; non-significant FR by lever by genotype interaction, $F_{(2,12)} = 0.321$, $p = 0.728$, NS).

Mice of both genotypes earned fewer reinforcers under higher FR requirements (Figure 4.5, significant effect of FR, $F_{(2,12)} = 16.54$, $p < 0.001$; non-significant FR by genotype interaction, $F_{(2,12)} = 0.052$, $p = 0.95$, NS; non-significant effect of genotype, $F_{(2,12)} = 0.35$, $p = 0.56$, NS).

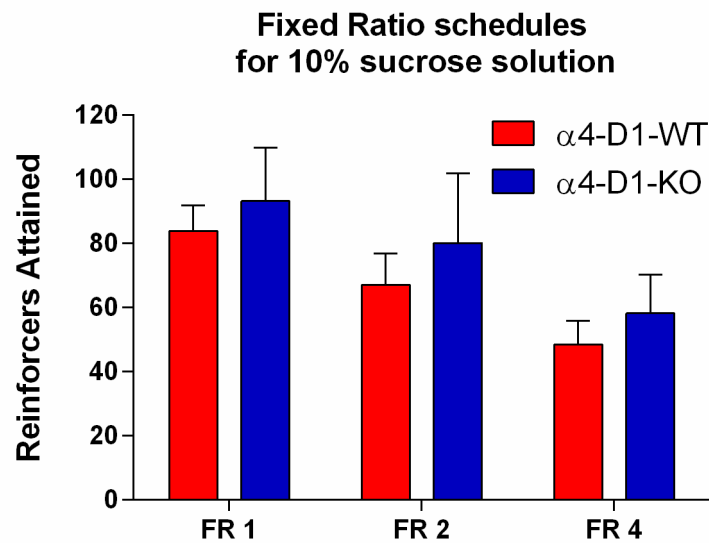


Figure 4.5. Graph FR Reinforcers earned over 60 minutes under FR1, FR2 and FR4 schedules of reinforcement. Both $\alpha 4$ -D1-WT ($n=8$) and $\alpha 4$ -D1-KO ($n=8$) mice earned a similar number of reinforcers at each ratio. Higher FR requirements reduced reinforcers earned by both genotypes. Error bars represent SEM.

4.3.4. Instrumental responding under Progressive Ratio schedules in $\alpha 4$ -D1-WT/ $\alpha 4$ -D1-KO mice

Mice responded significantly higher on the active lever than inactive lever and this was similar in both genotypes (Table 1.1, Figure 4.6; significant effect of lever, $F_{(1,13)} = 41.488$, $p < 0.001$; non-significant effect of genotype, $F_{(1,13)} = 3.43$, $p = 0.087$; non-significant genotype by lever interaction, $F_{(1,13)} = 3.43$, $p = 0.087$, NS). Both genotypes reached similar PR breakpoints before they stopped responding (non-significant effect of genotype, $F_{(1,13)} = 2.709$, $p = 0.124$, NS).

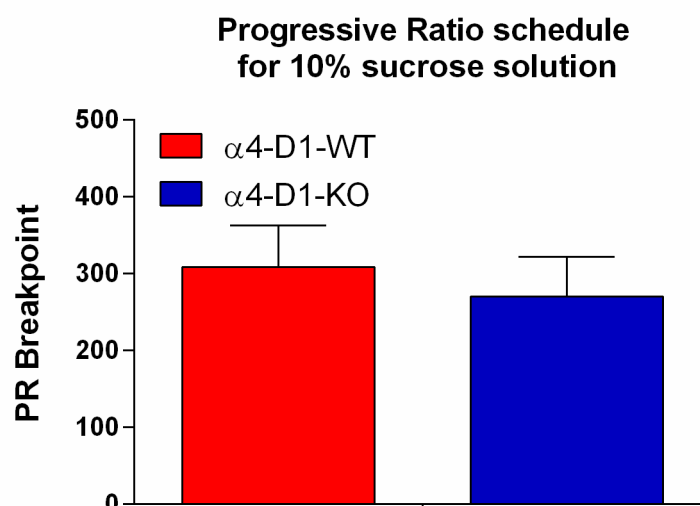


Figure 4.6. Progressive Ratio breakpoint (lever-presses) attained during 180-minute sessions by $\alpha 4$ -D1-WT (n=8) and $\alpha 4$ -D1-KO (n=8) mice. PR breakpoints were similar in both genotypes. Error bars represent SEM.

4.3.5. Cocaine potentiation of instrumental responding under Progressive Ratio schedules in $\alpha 4$ -D1-WT/ $\alpha 4$ -D1-KO mice

Mice responded significantly higher on the active lever than inactive lever and this was similar in both genotypes (Table 4.2; significant effect of lever, $F_{(1,12)} = 57.72$, $p < 0.001$; non-significant genotype by lever interaction, $F_{(1,12)} = 0.761$, $p = 0.4$, NS). Cocaine dose-dependently increased active lever presses to a greater extent than inactive lever presses (Table 4.2; significant main effect of dose, $F_{(3,10)} = 29.02$, $p < 0.001$; significant dose by lever interaction, $F_{(3,10)} = 40.85$, $p < 0.001$).

While both genotypes made similar active lever presses following I.P. saline (Figure 4.8; non-significant main effect of genotype $F_{(1,12)} = 0.085$, $p = 0.776$, NS) cocaine treatment increased active lever presses by $\alpha 4$ -D1-KO mice significantly more than in $\alpha 4$ -D1-WT mice (Figure 4.8; significant dose by genotype interaction $F_{(3,36)} = 7.97$, $p < 0.001$, significant dose by lever by genotype interaction, $F_{(3,36)} = 6.91$, $p < 0.001$).

Cocaine dose dependently increased PR breakpoints reached by mice of both genotypes (Figure 4.9; significant main effect of dose, $F_{(3,36)} = 18.44$, $p < 0.001$).

Both genotypes reached similar PR breakpoints following I.P. saline (non-significant main effect of genotype $F_{(1,12)} = 0.929$, $p = 0.35$, NS) cocaine treatment increased PR breakpoints reached by $\alpha 4$ -D1-KO mice significantly more than in $\alpha 4$ -D1-WT mice (Figure 4.9; significant dose by genotype interaction $F_{(3,36)} = 3.99$, $p < 0.05$).

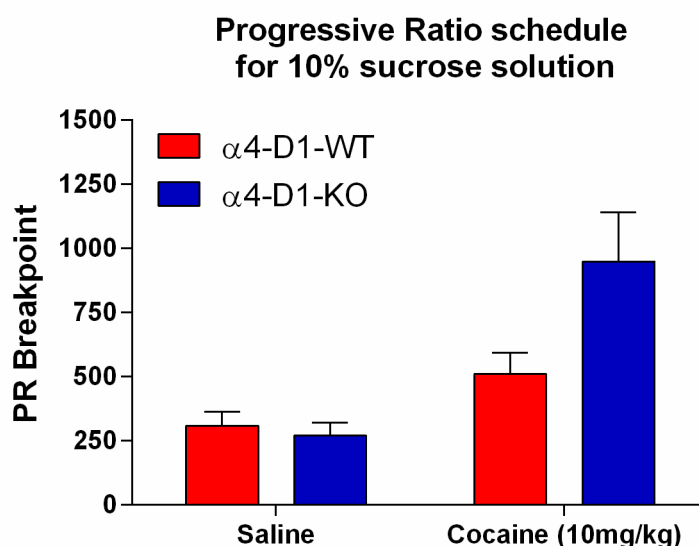


Figure 4.7. The effect of I.P. administration of cocaine (10mg/kg)/saline on PR breakpoints in 180-minute sessions for $\alpha 4$ -D1-WT ($n=8$) and $\alpha 4$ -D1-KO ($n=8$) mice. A challenge dose of cocaine significantly increased PR breakpoints in both genotypes ($p < 0.01$). Cocaine enhanced PR breakpoints significantly more in $\alpha 4$ -D1-KO than $\alpha 4$ -D1-WT mice ($p < 0.01$). Error bars represent SEM.

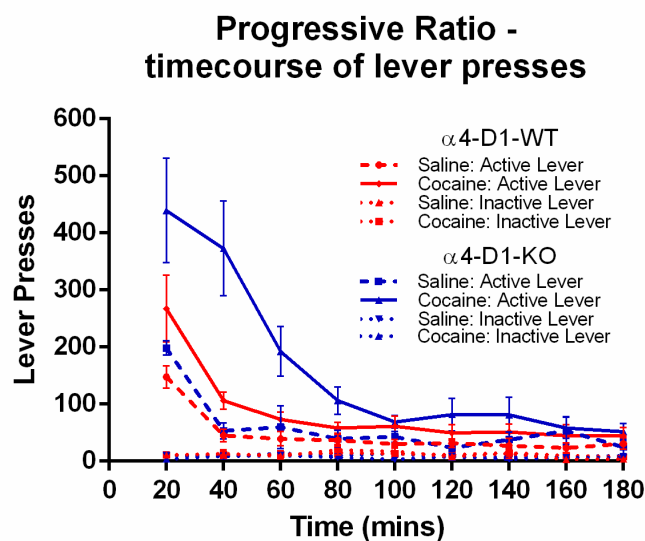


Figure 4.8. Timecourse of active and inactive lever presses over 60 minutes following I.P. injections of cocaine (10mg/kg) and saline in $\alpha 4$ -D1-WT (n=8) and $\alpha 4$ -D1-KO (n=8) mice. Both genotypes responded significantly more on the active than inactive lever. $\alpha 4$ -D1-KO mice responded significantly more on the active, but not inactive, lever following I.P. cocaine but not saline.

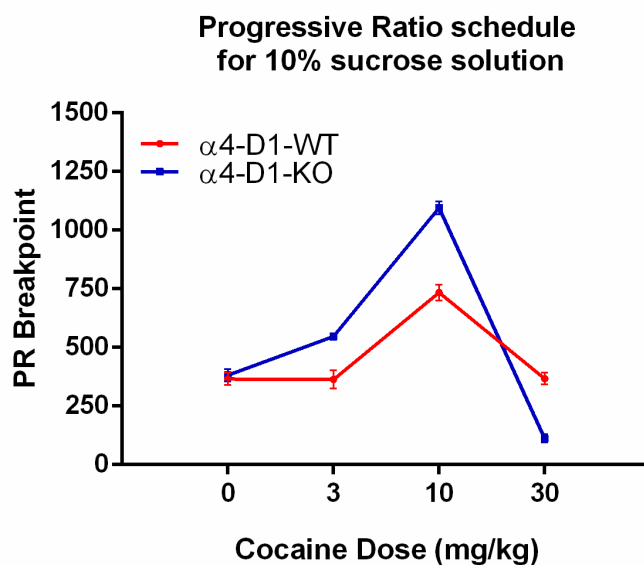


Figure 4.9. Effect of acute I.P. cocaine on PR breakpoints during 180-minute sessions in $\alpha 4$ -D1-WT (n=8) and $\alpha 4$ -D1-KO (n=8) mice. AT 10mg/kg cocaine increased PR breakpoints in both $\alpha 4$ -D1-WT (n=8) and $\alpha 4$ -D1-KO (n=8) mice. This enhancement was greater in $\alpha 4$ -D1-KO mice at 3 and mg/kg however they were impaired at 30mg/kg relative to $\alpha 4$ -D1-WT mice. Error bars represent SEM.

4.3.6. Instrumental responding under Fixed Ratio schedules in $\alpha 4$ -D2-WT/ $\alpha 4$ -D2-KO mice

Mice responded significantly higher on the active lever than inactive lever and this was similar in both genotypes (Table 1.1; significant effect of lever, $F_{(2,12)} = 59.76$, $p < 0.001$; non-significant effect of genotype, $F_{(2,12)} = 0.86$, $p = 0.37$, NS; non-significant genotype by lever interaction, $F_{(2,12)} = 0.87$, $p = 0.37$, NS).

Higher FR requirements elicited more active lever presses, but not inactive lever presses, from both genotypes (Figure 4.10; significant effect of FR, $F_{(2,12)} = 52.99$, $p < 0.001$; non-significant FR by genotype interaction, $F_{(2,12)} = 0.93$, $p = 0.41$, NS; significant FR by lever interaction $F_{(2,12)} = 54.59$, $p < 0.001$; non-significant FR by lever by genotype interaction, $F_{(2,12)} = 0.87$, $p = 0.37$, NS).

Mice of both genotypes earned fewer reinforcers under higher FR requirements (Figure 4.10; significant effect of FR, $F_{(2,12)} = 18.03$, $p < 0.001$; non-significant FR by genotype interaction, $F_{(2,12)} = 0.85$, $p = 0.451$, NS; non-significant effect of genotype, $F_{(2,12)} = 0.35$, $p = 0.56$, NS).

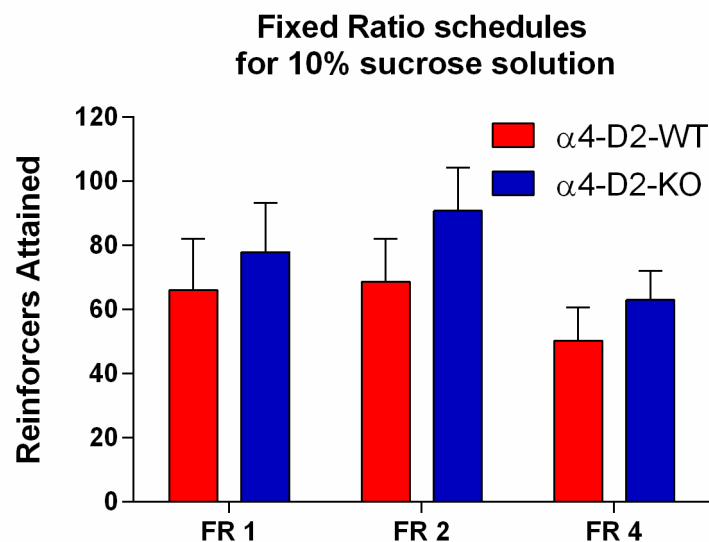


Figure 4.10. Graph FR Reinforcers earned over 60 minutes under FR1, FR2 and FR4 schedules of reinforcement. Both $\alpha 4$ -D2-WT ($n=8$) and $\alpha 4$ -D2-KO ($n=8$) mice earned a similar number of reinforcers at each ratio. Higher FR requirements reduced reinforcers earned by both genotypes.

4.3.7. Instrumental responding under Progressive Ratio schedules in $\alpha 4$ -D2-WT/ $\alpha 4$ -D2-KO mice

Mice responded significantly higher on the active lever than inactive lever and this was similar in both genotypes (Table 1.1; significant effect of lever, $F_{(1,13)} = 21.01$, $p < 0.001$; non-significant effect of genotype, $F_{(1,13)} = 0.82$, $p = 0.38$; non-significant genotype by lever interaction, $F_{(1,13)} = 0.5$, $p = 0.83$, NS). Both genotypes reached similar PR breakpoints before they stopped responding (Figure 4.11, non-significant effect of genotype, $F_{(1,13)} = 0.252$, $p = 0.62$, NS).

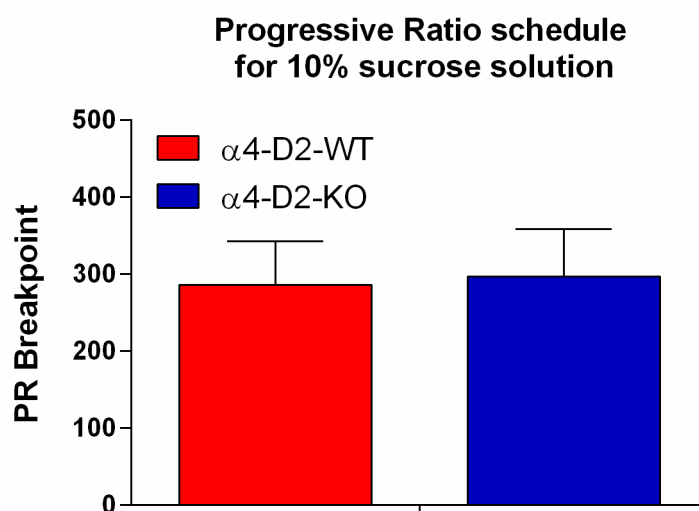


Figure 4.11. Progressive Ratio breakpoint (lever-presses) attained during 180-minute sessions by $\alpha 4$ -D1-WT ($n=8$) and $\alpha 4$ -D1-KO ($n=8$) mice. PR breakpoints were similar in both genotypes.

4.3.8. Cocaine potentiation of instrumental responding under Progressive Ratio schedules in $\alpha 4$ -D2-WT/ $\alpha 4$ -D2-KO mice

Mice responded significantly higher on the active lever than inactive lever and this was similar in both genotypes (Table 4.2; significant effect of lever, $F_{(1,12)} = 58.72$, $p < 0.001$; non-significant genotype by lever interaction, $F_{(1,12)} = 0.029$, $p = 0.87$, NS). Cocaine administration increased active lever presses to a greater extent than inactive lever presses (Table 4.2; significant main effect of drug, $F_{(3,10)} = 6.391$, $p < 0.05$; significant dose by lever interaction, $F_{(3,10)} = 7.59$, $p < 0.05$).

Cocaine potentiation of active lever responding was similar in both genotypes (Figure 4.13; non-significant main effect of genotype $F_{(1,12)} = 0.76$, $p = 0.4$, NS; non-significant drug by genotype interaction $F_{(3,36)} = 0.163$, $p = 0.69$, non-significant drug by lever by genotype interaction, $F_{(3,36)} = 0.059$, $p = 0.813$).

Cocaine administration increased PR breakpoints similarly in both genotypes (Figure 4.12; significant main effect of drug, $F_{(1,13)} = 17.53$, $p < 0.001$; non-significant main effect of genotype $F_{(1,12)} = 0.15$, $p = 0.7$, NS; non-significant dose by genotype interaction $F_{(3,36)} = 0.035$, $p = 0.85$).

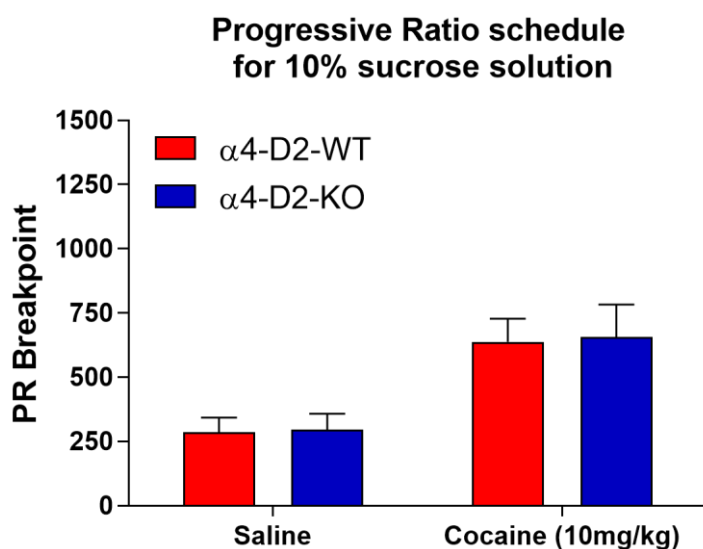


Figure 4.12. The effect of I.P. administration of cocaine (10mg/kg)/saline on PR breakpoints in 180-minute sessions for $\alpha 4$ -D2-WT ($n=8$) and $\alpha 4$ -D2-KO ($n=8$) mice. A challenge dose of cocaine significantly increased PR breakpoints ($p < 0.01$) similarly in both genotypes.

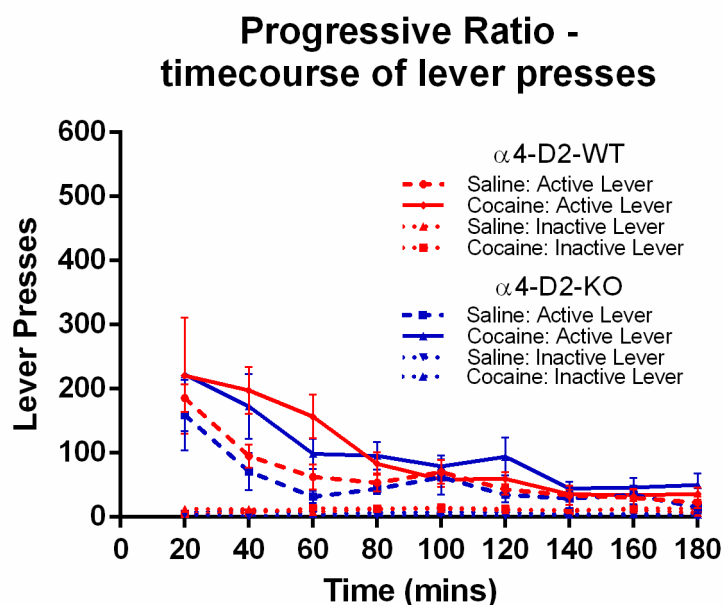


Figure 4.13. Timecourse of active and inactive lever presses over 60 minutes following I.P. injections of cocaine (10mg/kg) and saline in $\alpha 4$ -D2-WT (n=8) and $\alpha 4$ -D2-KO (n=8) mice. Both genotypes responded significantly more on the active than inactive lever following either saline or cocaine (10mg/kg) and this was similar in both genotypes.

Genotype	Schedule	Active lever	Inactive lever
$\alpha 4$ -D1-KO	FR1	93 \pm 18	3 \pm 0.4
	FR2	160 \pm 46	5.4 \pm 0.8
	FR4	233 \pm 51	4.4 \pm 0.9
	PR	466 \pm 69	62 \pm 28
$\alpha 4$ -D1-WT	FR1	83 \pm 8	4.8 \pm 0.6
	FR2	134 \pm 20	5.1 \pm 0.6
	FR4	194 \pm 30	5 \pm 0.9
	PR	855 \pm 203	27 \pm 9.4
$\alpha 4$ -D2-KO	FR1	77 \pm 15	4 \pm 0.7
	FR2	181 \pm 27	4.9 \pm 0.7
	FR4	252 \pm 36	4.9 \pm 0.8
	PR	482 \pm 168	30 \pm 9.9
$\alpha 4$ -D2-WT	FR1	66 \pm 17	4 \pm 0.5
	FR2	137 \pm 29	5.7 \pm 0.7
	FR4	201 \pm 44	4.6 \pm 1
	PR	597 \pm 99	99 \pm 26

Table 4.1. Baseline Active vs Inactive lever presses under each schedule of reinforcement for each genotype (Mean \pm SEM to two significant figures).

Genotype	PR + Drug	Active lever	Inactive lever
$\alpha 4$ -D1-KO	Saline	622 \pm 115	121 \pm 53
	Cocaine (3mg/kg)	849 \pm 51	144 \pm 47
	Cocaine (10mg/kg)	1578 \pm 87	61 \pm 15
	Cocaine (30mg/kg)	184 \pm 66	111 \pm 33
$\alpha 4$ -D1-WT	Saline	569 \pm 102	83 \pm 12
	Cocaine (3mg/kg)	502 \pm 139	55 \pm 18
	Cocaine (10mg/kg)	1041 \pm 121	63 \pm 9
	Cocaine (30mg/kg)	513 \pm 97	10 \pm 8
$\alpha 4$ -D2-KO	Saline	482 \pm 79	23 \pm 4
	Cocaine (10mg/kg)	904 \pm 77	58 \pm 13
$\alpha 4$ -D2-WT	Saline	597 \pm 37	99 \pm 10
	Cocaine (10mg/kg)	882 \pm 55	102 \pm 13

Table 4.2. Active vs Inactive lever presses under PR schedules following different drug treatments (Mean \pm SEM).

4.4. Discussion

The present data demonstrate that conditionally deleting GABA_A $\alpha 4\beta\delta$ receptors on D1-MSNs, facilitates cocaine-potentiation of locomotor activity. This agrees with multiple studies in which genetic or pharmacological methods to activate D1 MSNs increased cocaine-induced locomotion (Schindler and Carmona, 2002; Bachtell *et al.*, 2005b; A. V Kravitz *et al.*, 2010).

Notably we did not observe any differences in baseline locomotor activity in $\alpha 4$ -D1-KO mice. We may have expected an increase as in other studies activation of D1 receptors potentiated locomotor activity in the absence of cocaine (Dreher and Jackson, 1989). We therefore suggest that D1 MSNs which express $\alpha 4$ are a sub-population which mediate the effects of psychostimulants but are not normally involved in initiation of locomotor activity (see Chapter 1). This may be due to the relatively high expression of $\alpha 4$ in the NAc, which is known to mediate the locomotor activating properties of cocaine, relative to the rest of the striatum (Costall *et al.*, 1977; Robinson and Berridge, 1993; Campbell *et al.*, 1997; Schwarzer *et al.*, 2001).

Further we have identified a dissociation between the effects of $\alpha 4\beta\delta$ receptors on D1 and D2 MSNs on cocaine-induced locomotor activity since deletion of $\alpha 4$ in D2 MSNs had no such effect. Based on previous studies using injections of D2 agonists in the NAc we might have expected an increase in cocaine potentiated

locomotor activity (Bachtell *et al.*, 2005b), however those effects were smaller than those produced by D1 agonists therefore deletion of $\alpha 4\beta\delta$ GABA_ARs may not have a sufficiently strong effect on D2 MSNs to replicate such findings. Given that systemic administration of D2 agonists results in mixed outcomes (Schindler and Carmona, 2002; Stuchlik *et al.*, 2007) it is also possible that more anatomically targeted D2 manipulations are required to due to confounding effects.

Previously, deletion of either GABA_AR $\alpha 4$ -subunits or δ -subunits, often paired in extrasynaptic $\alpha 4\beta\delta$ GABA_ARs, had no influence on baseline locomotion (Herd *et al.*, 2009; Macpherson, 2013) and constitutive GABA_AR $\alpha 4$ -subunit knockout mice showed no difference from wildtype mice in locomotor activity following various doses of cocaine. There is however evidence that when pharmacologically activated $\alpha 4\beta\delta$ receptors oppose the locomotor stimulating effects of cocaine. Intra-NAc injections of the agonist THIP attenuated cocaine-induced locomotor activity and this effect was abolished in $\alpha 4$ -KO (Macpherson, 2013).

These data suggest that $\alpha 4$ -GABA_ARs are not important for the initiation of locomotion, but their activation is able to attenuate baseline and cocaine-potentiated locomotor activity. Our results indicate that this is most likely mediated by activation of $\alpha 4\beta\delta$ receptors on D1 MSNs. This could be fully confirmed by intra-NAc administration of THIP to D1/D2 $\alpha 4$ conditional knockout mice in larger cocaine-dose-response study of locomotor activity.

Cocaine potentiates locomotor activity by prolonging the action of dopamine which is released in the NAc by projections from the VTA (Costall *et al.*, 1984). This subsequently increases the sensitivity of MSNs to glutamatergic inputs which they receive from other areas such as the frontal cortex, amygdala and hippocampus (O'Donnell and Grace, 1995). We hypothesise that deletion of $\alpha 4$ -GABA_ARs will attenuate tonic inhibition on D1 MSNs in the NAc (Maguire *et al.*, 2014) consequently increasing the impact of glutamatergic excitation which is facilitated by cocaine-enhanced synaptic dopamine.

We did not observe difference in cocaine potentiation of locomotor activity or instrumental responding in the $\alpha 4$ constitutive knockout - i.e. it predominates

over the $\alpha 4$ -D1-KO phenotype. This implies that concurrent disinhibition of D2 MSNs, and/or $\alpha 4$ -containing interneuron populations that express D2, is in some way compensatory for disinhibition of D1 MSNs; possibly through opposing effects on locomotor activity. This is supported by studies that observed decreased cocaine-potentiated locomotor activity following activation of D2 MSNs in the NAc by optogenetic stimulation or DREADDs (Chandra *et al.*, 2013; Zhu, Ottenheimer and DiLeone, 2016). If disinhibition of D1 and D2 MSNs has opposite effects we would expect that $\alpha 4$ -D2-KO mice should show reduced cocaine-potentiation of locomotor activity and instrumental responding, which they do not. However, absence of $\alpha 4$ -GABA_AR-mediated inhibition in D2-MSNs may override the inhibition of D2-MSNs normally caused by cocaine-induced dopamine release, resulting in locomotor activity of $\alpha 4$ -D2-KO mice remaining unaffected by cocaine.

As $\alpha 4$ -GABA_ARs on D1 MSNs oppose cocaine's stimulant effects this may act as a homeostatic control to prevent excessive neuronal excitation by dopamine. The $\alpha 4$ subunit is epigenetically upregulated following high dose or chronic cocaine administration and this occurs preferentially in D1 MSNs (Heiman *et al.*, 2008) indicating this may also act as a long-term, adaptive homeostatic mechanism. Further experiments on locomotor sensitisation in $\alpha 4$ -D1-KO mice may provide insight on this possibility (see chapter 5).

We have also demonstrated that conditionally deleting $\alpha 4$ -GABA_A receptors on D1 MSNs facilitates cocaine-potentiation of instrumental responding for natural rewards under a PR schedule, as it does with locomotor activity. Again, we did not observe any differences in baseline instrumental responding in $\alpha 4$ -D1-KO mice. This contrasts with previous studies where activation of D1 or D2 MSNs systemically or in the NAc facilitated responding under PR schedules reinforced by food or sucrose (Aberman, Ward and Salamone, 1998; Barbano, Le Saux and Cador, 2009). This implies that, as with locomotor activity, $\alpha 4$ -GABA_AR containing MSNs are a subset of neurons which mediate effects of psychostimulants but do not alter behaviour under normal conditions.

The similar pattern of effects on locomotor activity and instrumental responding we observed in $\alpha 4$ -D1-KO mice indicates a 'general increase in approach-investigation' behaviour may underlie behavioural outcomes in both tests

(Ikemoto and Panksepp, 1999). The alternative hypothesis is that cocaine increases efficacy of primary reinforcers in $\alpha 4$ -D1-KO mice. This is unlikely as in previous studies similar doses of cocaine reduced preference for sucrose (Balopole, Hansult and Dorph, 1979) and baseline responding was unaffected which indicated changes in GABAergic activity do not modulate appetite (Zhang, Balmadrid and Kelley, 2003). Whilst $\alpha 4$ -D1-KO show greater enhancement of PR breakpoints following 10mg/kg cocaine they are more severely impaired following 30mg/kg indicating that they are more prone to stereotypy at high doses due to hypersensitivity to the stimulant effects of cocaine.

Several previous studies identified that psychostimulant potentiation of both locomotor and PR instrumental responding are mediated by the NAc shell and similarly modulated by dopamine agonists which links these behaviours to a common neural substrate and mechanism (Zhang, Balmadrid and Kelley, 2003; Bachtell *et al.*, 2005b). We conclude that increased cocaine potentiation of PR responding in $\alpha 4$ -D1-KO mice is likely due to the enhanced locomotor stimulant effects of cocaine.

These results are significant to the interpretation of previous CRf experiments using constitutive and conditional $\alpha 4$ knockout mice. There is a dissociation between locomotor activity/instrumental responding and CRf in the different transgenic $\alpha 4$ lines. $\alpha 4$ -D1-KO mice show facilitation of locomotor activity and instrumental responding which was absent in constitutive $\alpha 4$ -KO or $\alpha 4$ -D2-KO mice. Conversely $\alpha 4$ -KO or $\alpha 4$ -D2-KO mice show enhancement baseline and cocaine potentiated CRf which is absent in $\alpha 4$ -D1-KO mice (Macpherson, 2013).

Deletion of $\alpha 4$ in D2 MSNs does not seem to alter primary reinforcer efficacy as measured by fixed and progressive ratio responding, nor does it increase the locomotor stimulating effects of cocaine as measured in the dose-responses for locomotor activity and progressive ratio schedules. This supports the hypothesis that removal of tonic inhibition of D2 MSNs enhances efficacy of secondary reinforcers and further enhances cocaine's potentiation of CRf independently of its locomotor activating properties (Macpherson, 2013).

To conclude, deletion of GABA_AR $\alpha 4$ -subunits on D1 or D2 MSNs had no effect on baseline locomotor activity or instrumental responding. However, deletion of $\alpha 4$ -

GABA_ARs on D1, *but not* D2, MSNs increased cocaine's ability to potentiate both behaviours. We interpret this as an enhanced general stimulant effect of cocaine in $\alpha 4$ -D1-KO mice which is not present in $\alpha 4$ -KO or $\alpha 4$ -D2-KO mice. These data indicate that $\alpha 4$ -GABA_ARs specifically on D1 MSNs provide an efficacious target for control of the stimulant properties of cocaine. Investigation into the effects of GABA_AR $\alpha 4$ -subunit deletion in D1/D2 MSNs on locomotor sensitisation to cocaine in further experiments may elucidate a role of $\alpha 4$ in adaptive changes following chronic cocaine administration.

Chapter 5

The role of GABA_A $\alpha 4$ Receptor subunits on D1 and D2 expressing neurons in mediating behavioural sensitisation to cocaine

5.1. Introduction

Behavioural sensitisation is a phenomenon whereby repeated administration of a drug results in increased stimulant behavioural effects (Tilson and Rech, 1973; Segal and Mandell, 1974; Browne and Segal, 1977; Bailey and Jackson, 1978; Hirabayashi and Alam, 1981; Leith and Kuczenski, 1982; Robinson and Becker, 1986). This has been reported following administration of many drugs, including cocaine (Post et al., 1987; Kalivas and Stewart, 1991), amphetamine (Robinson and Becker, 1986; Cador et al., 1999), opiates (Babbini and Davis, 1972; Shuster et al., 1975), nicotine (Benwell and Balfour, 1992; Kita et al., 1992) and ethanol (Cunningham and Noble, 1992; Phillips et al., 1997).

Robinson and Berridge (1993) have argued that repeated exposure also leads to sensitisation to the incentive motivational properties of drugs. Under this 'incentive sensitisation' model neural substrates mediating the attribution of incentive salience, termed 'wanting', are sensitised by repeated drug exposure, whereas substrates which mediate the hedonic experience of a drug, termed 'liking', remain unsensitised or diminished (Robinson and Berridge, 2008). This is supported by evidence that sensitisation with amphetamine, cocaine, morphine or ethanol has facilitated the subsequent acquisition of self-administration or conditioned place preference (CPP) produced by the same drug, or a different drug (Lett, 1989; Horger, Shelton and Schenk, 1990; Piazza et al., 1990; Mendrek, Blaha and Phillips, 1998; Hoshaw and Lewis, 2001; Camarini and Hodge, 2004; McDaid et al., 2005).

Psychostimulants are particularly robust in their ability to induce behavioural sensitisation and the neuroadaptations which underlie it. Behavioural sensitisation to amphetamine was demonstrated to persist undiminished for over a year (Robinson and Berridge, 1993). Studies exploring the neurobiological basis of behavioural sensitisation to cocaine have largely focussed on the mesolimbic dopamine system due to the established role of this system in

mediating the locomotor activating properties of cocaine (Pierce & Kalivas, 1997; Robinson & Berridge, 1993). It is well established that locomotor activity is enhanced by drugs that facilitate transmission at dopamine synapses, moreover it is reduced by dopamine antagonists or lesions of dopaminergic systems (Kelly, Seviour and Iversen, 1975; Kelly and Iversen, 1976; Wachtel and Anden, 1978; Fray et al., 1980).

When dopamine agonists were infused directly into various regions of the rat forebrain the behavioural outcomes suggested that locomotor stimulation was primarily mediated by the Nucleus Accumbens (NAc), and stereotyped behaviours from the Dorsal Striatum (Costall *et al.*, 1977; Campbell *et al.*, 1997). The NAc has been found to be critical in mediating behavioural sensitisation to drugs including psychostimulants, ethanol and morphine following repeated administration (Tilson and Rech, 1973; Segal and Mandell, 1974; Cador, Taylor and Robbins, 1991; Kalivas and Duffy, 1993; Hoshaw and Lewis, 2001). During development of cocaine-sensitisation, repeated intermittent cocaine administration elevated basal extracellular level of dopamine within the NAc (Di Chiara and Imperato, 1988; Kalivas and Duffy, 1990). In addition, D1 dopamine receptors in the NAc show enhanced sensitivity to dopamine following repeated cocaine administration (Henry, Greene and White, 1989; Henry and White, 1991). Lesions of the NAc shell attenuated of the induction of, but not expression of, behavioural sensitisation to cocaine (Todtenkopf *et al.*, 2002; Todtenkopf, Stellar and Melloni, 2002), and infusions of cocaine or amphetamine to the NAc shell, but not core, induced behavioural sensitisation (Pierce and Kalivas, 1997).

As outlined in earlier chapters, the majority of the striatum (~95%) is made up of GABAergic MSNs which can be divided into two subtypes, D1R- and D2R-expressing MSNs (Kemp and Powell, 1971; Gerfen *et al.*, 1990). Recent evidence indicates we must exercise caution in referring to D1 and D2 populations as the 'direct' or 'indirect' pathways within the ventral striatum (Kupchik *et al.*, 2015). A large body of research has elucidated distinct and often opposing functions of D1 and D2 neuronal populations, sometimes referred to as the go/no-go pathways due to their roles in action initiation/inhibition (Surmeier, 2013).

D1 and D2 neurons of the ventral striatum/NAc have been implicated in mediating the acute and chronic effects of drugs of abuse (Lobo and Nestler, 2011). Many studies have specifically manipulated D1 or D2 striatal populations to examine their roles in behavioural sensitisation. Pharmacological manipulations have had mixed effects depending on type of agonist/antagonist, method and timing of administration (Mazurski and Beninger, 1991; Lobo and Nestler, 2011). It was proposed that genetically targeted manipulations may be able to more accurately dissociate these pathways (Durieux, Schiffmann and de Kerchove d'Exaerde, 2011; Lobo and Nestler, 2011).

Blockade of neurotransmission via viral expression of tetanus toxin in either D1- or D2-MSNs of the NAc attenuated the locomotor activating effects of acute cocaine injection in mice (Hikida *et al.*, 2010). Furthermore, blockade of D1 MSNs attenuated sensitisation to cocaine over six drug exposures, whereas blockade in D2-MSNs slightly delayed the acquisition of sensitisation. Importantly, these phenotypes were normalized by reversal of the blockade indicating that D1/D2 neurons mediate expression rather than acquisition of cocaine sensitisation. Designer Receptors Activated by Designer Drugs (DREADDs) were used to inhibit D1 neurons or D2 neurons during a regimen of amphetamine treatment (six drug exposures) which induced robust behavioural sensitisation in controls (Ferguson *et al.*, 2011). Neither affected acute response to amphetamine in the first session, but inactivation of D2 neurons resulted in increased sensitization whilst inactivation of D1 neurons had no effect on the acquisition of sensitisation. Notably, in this experiment response to a challenge dose after a one-week withdrawal period was reduced or enhanced in the D1- or D2-DREADD groups respectively even in the absence of DREADD activation. This indicates that activation of D1 or D2 neurons during sensitisation facilitates or opposes subsequent expression of psychostimulant sensitisation respectively.

A knock-in mutation which diminished NMDA receptor (NMDAR) conductance in D1 MSNs abolished acquisition of cocaine-induced CPP and locomotor sensitization, highlighting the necessity for NMDA signalling in D1 MSNs for the rewarding and sensitizing effects of cocaine (Heusner and Palmiter, 2005). Subsequently, it was found that deletion of NMDARs in D2 MSNs normalized sensitization in mice already lacking NMDARs in D1 receptors (Beutler *et al.*,

2011). This demonstrates that a balance of D1 and D2 neuronal activity is critical for establishing sensitisation. Conditional knock-out of the acetylcholine receptor 4 (M4) , an inhibitory metabotropic receptor, in D1 neurons increased their firing and resulted in accelerated acquisition of sensitisation to both cocaine and amphetamine and increased overall locomotor response to those drugs across all 6 sessions (Jeon *et al.*, 2010). Optogenetic stimulation of D1, but not D2, neurons enhanced locomotor activity in cocaine-sensitised but not naive mice (Lobo *et al.*, 2010). Although effects vary slightly depending on the type and location of genetic manipulations, together they indicate that activation of D1 MSNs within the NAc mediate behavioural sensitisation to psychostimulants while activation of D2 in the NAc neurons may oppose it.

Chronic cocaine administration results in physiological adaptations of neurons in the direct and indirect striatal pathways. For example, long-term, intermittent cocaine administration increased spine density on dendrites of MSNs in the NAc (Robinson & Kolb, 1999; Robinson *et al.*, 2001). A number of signalling molecules are known to be induced by psychostimulants including FosB (Hope *et al.*, 1994), cFos (Robertson *et al.*, 1991) and ERK (Bertran-Gonzalez *et al.*, 2008). The transcription factor protein Δ FosB, is thought to act as an important molecular “switch” in the transition from drug abuse to addiction (Nestler *et al.* 2001; Nestler, 2005). Mice with elevated Δ FosB in the NAc exhibited similar phenotype to cocaine-sensitised mice, including increased locomotor response to cocaine, as well as increased self-administration and motivation for cocaine (Kelz *et al.*, 1999; Nestler *et al.*, 2001). Conversely, blocking the build-up of Δ FosB in mice during a regimen of cocaine exposure reduced these behaviours (Nestler *et al.*, 2001; Nestler, 2004). Repeated cocaine exposure preferentially induces Δ FosB in D1 neurons specifically (Hope *et al.*, 1994).

Activation of the proto-oncogene cFos has been widely characterised and used to map neural activity (Sheng and Greenberg, 1990). In rats there is an increase in cFos expressing neurons in the NAc following behavioural sensitisation to cocaine (Crombag *et al.* 2002). Further, following sensitisation cFos is increased preferentially in D1 Neurons (Young *et al.* 1991, Bertran-Gonzalez *et al.* 2008; Guez-Barber *et al.* 2011). Manipulation of signalling molecules in D1 or D2 populations also differentially affects cocaine sensitisation. Conditional deletion

of cFos in D1 neurons blunted dendritic remodelling and attenuated expression of cocaine sensitisation (Zhang *et al.*, 2006). Similarly, D1 cell specific deletion of DARPP-32, a dopamine activated signalling molecule, diminished sensitisation whereas deletion of DARPP-32 from D2 neurons led to enhanced acquisition of cocaine sensitisation (Bateup *et al.*, 2008). Conversely deletion of BDNF in D1 MSNs enhanced sensitisation and cFos expression in the NAc Shell whilst deletion in D2 MSNs attenuated sensitisation, and both phenotypes were rescued by restoring BDNF expression in the NAc using viral vectors (Lobo *et al.*, 2010).

Chronic cocaine administration alters GABA receptor and other ion channel subunits specifically in D1 neurons (Heiman *et al.*, 2008). It is thought that repeated exposure to cocaine induces changes in GABA systems, resulting in a dysregulation of the neural circuitry mediating behavioural responses to drugs (Koob and Le Moal, 2001; Koob and Volkow, 2010). Ex-vivo analysis of striatal slices from cocaine-sensitised rats demonstrated that behavioural sensitisation to cocaine decreased pre- and post-synaptic GABA transmission (Jung *et al.*, 1999), and reduced the function of GABA_ARs (Peris, 1996). Muscimol binding was unaffected indicating that overall GABA_AR levels remain similar (Jung and Peris, 2001). Conversely, following withdrawal from cocaine sensitisation or repeated cocaine administration in mice, cocaine challenges increase GABA transmission in the mPFC (Jayaram and Steketee, 2005) and NAc (Xi *et al.*, 2003).

The GABA_AR α 2-subunit is the predominant alpha subunit within the NAc and thus is likely to play an important role in mediating behavioural responses to cocaine (Schwarzer *et al.*, 2001). Following amphetamine-sensitisation a decrease in GABA_AR α 2-subunits is reported within the NAc shell and core (Zhang *et al.*, 2006). Furthermore, targeted deletion of the GABA_AR α 2-subunit blocks the ability of cocaine to induce behavioural sensitisation (Dixon *et al.*, 2010), an effect subsequently demonstrated to be mediated within the NAc. Selective activation of α 2-containing GABA_ARs within the NAc using intracranial infusions of the atypical benzodiazepine Ro 15-4513, were sufficient to induce behavioural sensitisation in α 2(H101R) mutant mice (Dixon *et al.*, 2010).

Involvement of the GABA_AR α 4 subunit in mediating behavioural sensitisation to cocaine has also been suggested. Systemic administration of THIP, an agonist

selective for extrasynaptic $\alpha 4$ -GABA_ARs, blocks both the acquisition and expression of behavioural sensitisation to amphetamine (Karler et al., 1997). With regard to D1 and D2 pathway involvement, $\alpha 4$ is upregulated preferentially in D1 neurons following repeated or high dose cocaine administration (Heiman et al., 2008).

In previous experiments we did not find any effect of constitutive deletion of the $\alpha 4$ subunit on behavioural sensitisation to cocaine (Macpherson, 2013), although systemic THIP suppressed locomotor sensitisation in $\alpha 4$ -WT but not $\alpha 4$ -KO mice. We hypothesise that the lack of change in behavioural sensitisation to cocaine following global knockout of GABA_A $\alpha 4$ subunits could be explained by the dissociable effects in dopamine D1- or D2-expressing neurons cancelling each other out and resulting in no overall change, as in the case of NMDAR receptor deletion (Beutler et al., 2011).

Here we used immunohistochemical/in-situ-hybridisation analysis of cFos expression in D1 and D2 MSNs to examine the effects of acute and chronic cocaine administration on neural activity in the NAc of $\alpha 4$ -KO mice compared with wildtypes. To elucidate the role of $\alpha 4$ -GABA_ARs D1/D2 expressing neurons in behavioural sensitisation we examined the effects of D1- or D2-specific deletion of $\alpha 4$ containing GABA_A receptors on behavioural sensitisation to cocaine. We also performed immunohistochemical analysis of cFos expression to investigate the effects of these manipulations on neural activity.

5.2. Materials and Methods

5.2.1. Animals

All mice were generated on C57BL/6J background strain. Mice homozygous for a null-mutation of the *gabra4* gene ($\alpha 4$ -KO) and homozygous wild-type mice ($\alpha 4$ -WT) were generated by breeding heterozygous mice carrying one copy each of both the knock-out and wild-type allele ($\alpha 4$ -Het) as described previously in chapter 2 (Chandra et al., 2006; Maguire et al., 2014).

As described in chapter 2 we produced conditional knock-out lines by crossing 'floxed' $\alpha 4$ mice [strain name; B6.129-Gabra4^{tm1.2Geh/J}; Jackson Laboratory] (Chandra et al., 2006) with BAC D1-CRE [MMRRC strain B6.FVB(Cg)-Tg(Drd1a-

cre)EY266Gsat/Mmucd] or BAC D2-CRE [MMRRC strain B6.FVB(Cg)-Tg(Drd2-cre)ER44Gsat/ Mmucd] (Gong *et al.*, 2007) to result in cre-mediated deletion of $\alpha 4$ in either D1 or D2 expressing cells of the offspring. Mice hemizygous for the BAC D1-Cre transgene and homozygous for the 'floxed' $\alpha 4$ transgene were bred with homozygous 'floxed' $\alpha 4$ mice to produce $\alpha 4$ -D1-KO and $\alpha 4$ -D1-WT littermates whilst mice hemizygous for the BAC D2-Cre transgene and homozygous for the 'floxed' $\alpha 4$ transgene were bred with homozygous 'floxed' $\alpha 4$ mice to produce $\alpha 4$ -D2-KO and $\alpha 4$ -D2-WT littermates as previously described (Maguire *et al.*, 2014).

Male and female mice weighing between 20-30g and aged between 2-4 months, were housed in groups of 2-3, with food and water available *ad libitum*. During the habituation period and experiment a reversed 12hr light/dark cycle was used (lights on at 11:00 P.M.) with holding room temperature maintained at $21 \pm 2^\circ\text{C}$ and humidity $50 \pm 5\%$.

5.2.2. Drugs

Cocaine Hydrochloride was obtained from Macfarlan Smith (Edinburgh, UK). Cocaine was dissolved in 0.9% saline, and administered IP at an injection volume of 10 ml/kg.

5.2.3. Apparatus

Behavioural sensitisation was measured by recording locomotor activity in 16 annular black Perspex runways, (diameter 24cm, annula width 6.5cm), placed atop a clouded Perspex sheet on an elevated frame. A digital camera positioned beneath the sheet captured the silhouettes of the boxes' edges and the mice within them, which was then relayed to a computer to be recorded. A MatLab (MathWorks, Cambridge, UK) video analysis programme and Excel macro converted the video data into a measure of the distance travelled in metres.

5.2.4. Behavioural Sensitisation to Cocaine (10mg/kg) Procedure

(Figure 5.1, 5.2) Prior to testing, mice were habituated to the locomotor runways in two sessions. On the first day mice were habituated to the equipment for 60 minutes, then, on the second day, mice received IP injections of saline followed by a 60-minute habituation session. Subsequently, mice received repeated, intermittent treatment of either cocaine (10mg/kg) or saline for 10 consecutive daily sessions. Activity was recorded for 60 minutes each

session. Before every session mice were allowed to habituate to the runways for 30 minutes before being returned to their homecage. After 5 minutes, mice were dosed with either cocaine (10mg/kg) or saline and returned to runways.

5.2.5. Conditioned Activity

(Figure 5.2) After 10 days of cocaine or saline treatment mice were placed in the locomotor runways as described above, with all animals receiving 10ml/kg saline injections. Activity was recorded for 60 minutes.

5.2.6. Response to a cocaine (20mg/kg) challenge in sensitised vs non-sensitised animals

(Figure 5.1, 5.2) Cocaine-sensitised and saline-treated mice were divided into two groups, counterbalanced by sex and genotype, half receiving cocaine (20mg/kg) and the other half receiving saline directly prior to initiation of the locomotor test. Activity was recorded for 60 minutes each session.

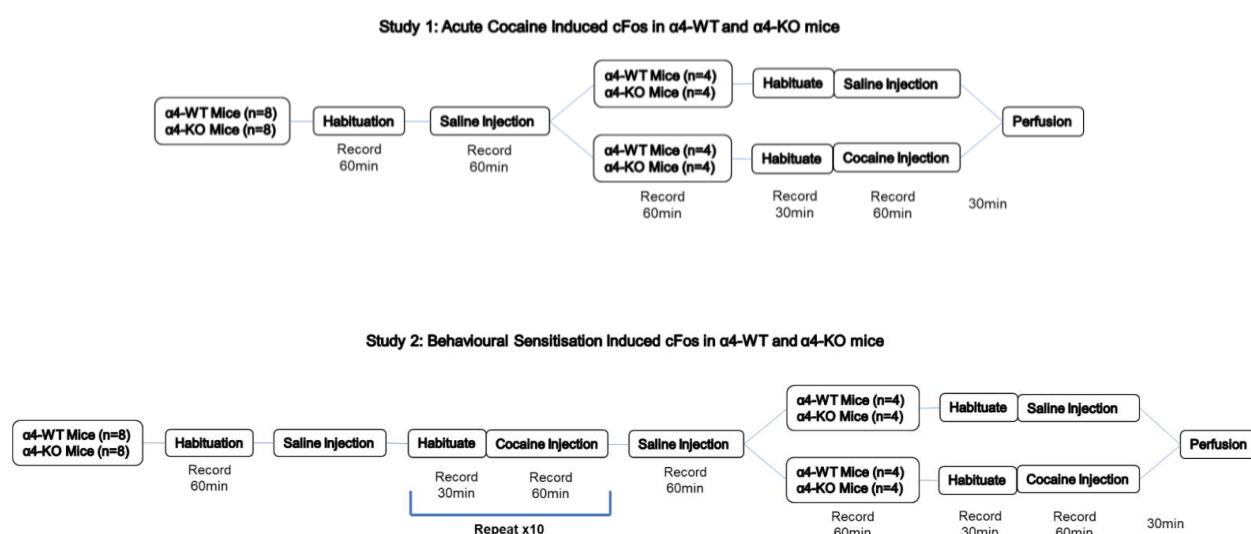


Figure 5.1. Experimental Design for studies 1 and 2. Investigating the effects of 20mg/kg cocaine challenge compared with saline in naive (study 1) or cocaine-sensitised (study 2) α 4-KO and α 4-WT mice.

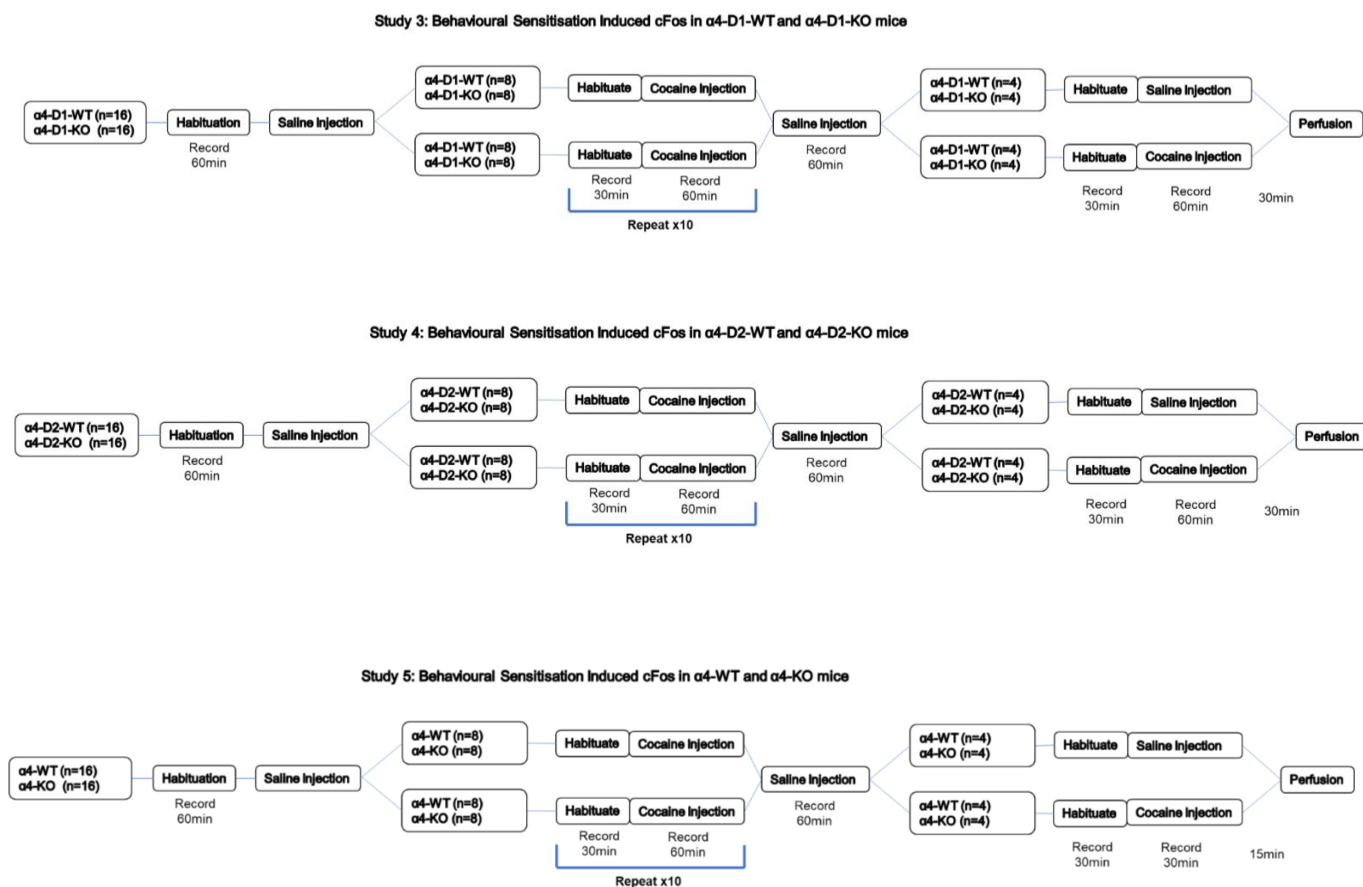


Figure 5.2. Design of studies which compare behavioural sensitisation to cocaine (10,g/kg) in constitutive or D1/D2 selective $\alpha 4$ GABA_AR receptor subunit knockout mice compared with respective wildtype controls. Includes saline-treated control groups which are used to directly compare sensitised and non-sensitised animals with a challenge of cocaine (20mg/kg) or saline in the final session.

5.2.7. Design of Experiments

Study 1. Both $\alpha 4$ -WT (n=8) and $\alpha 4$ -KO (n=8) mice were habituated to locomotor runways in a 60minute session and habituated to saline injection in another 60-minute session (Figure 5.1). On the challenge day mice received either IP saline injection (n=4 per genotype) or IP cocaine (20mg/kg) (n=4 per genotype).

Study 2. Both $\alpha 4$ -WT (n=8) and $\alpha 4$ -KO (n=8) mice underwent the behavioural sensitisation procedure as described (Figure 5.1) with all mice receiving daily 10mg/kg cocaine injections. On the challenge day mice received either IP saline injection (n=4 per genotype) or IP cocaine (20mg/kg) (n=4 per genotype).

Study 3. Both $\alpha 4$ -D1-WT (n=16) and $\alpha 4$ -D1-KO (n=16) mice underwent the behavioural sensitisation procedure as described (Figure 5.2) with all half of the mice receiving daily saline injections (n=8 per genotype) and half cocaine (n=8 per genotype). On the challenge day cocaine-sensitised mice received either IP saline injection (n=4 per genotype) or IP cocaine (20mg/kg) (n=4 per genotype) and saline-treated mice received either IP saline injection (n=4 per genotype) or IP cocaine (20mg/kg) (n=4 per genotype).

Study 4. Both $\alpha 4$ -D2-WT (n=16) and $\alpha 4$ -D2-KO (n=16) mice underwent the behavioural sensitisation procedure as described (Figure 5.2) with all half of the mice receiving daily saline injections (n=8 per genotype) and half cocaine (n=8 per genotype). On the challenge day cocaine-sensitised mice received either IP saline injection (n=4 per genotype) or IP cocaine (20mg/kg) (n=4 per genotype) and saline-treated mice received either IP saline injection (n=4 per genotype) or IP cocaine (20mg/kg) (n=4 per genotype).

Study 5. Both $\alpha 4$ -WT (n=16) and $\alpha 4$ -KO (n=16) mice underwent the behavioural sensitisation procedure as described (Figure 5.2) with half of the mice receiving daily saline injections (n=8 per genotype) and half cocaine (n=8 per genotype). On the challenge day cocaine-sensitised mice received either IP saline injection (n=4 per genotype) or IP cocaine (20mg/kg) (n=4 per genotype) and saline-treated mice received either IP saline injection (n=4 per genotype) or IP cocaine (20mg/kg) (n=4 per genotype).

5.2.8. Immunohistochemistry

At 90 minutes following the 20mg/kg cocaine administration mice were euthanised by IP injection of Sodium Pentobarbital (200mg/kg, 10ml/kg). Mice brains were perfused via the aorta with 25ml (5 minutes of 5ml/min) of phosphate buffer solution (PBS) followed by 75ml (15 minutes of 5ml/min) of 4% paraformaldehyde (PF) (Sigma-Aldrich, St. Lois, MO, USA) in PBS. After perfusion, brains were removed and post-fixed overnight in 4% PF in PBS at 4°C, then transferred into 30% sucrose solution in PBS and left for 3 days at 4°C to cryoprotect. Coronal sections (30 μ m thick) were cut using a cryostat and collected in PBS-azide.

Free floating sections were washed 3 times in PBS for 10 minutes then incubated in blocking solution (3% Normal Goat Serum, Vector Labs, in PBS-T) for 1 hour with gentle agitation. Sections were then immediately incubated overnight in rabbit anti-cFos polyclonal primary antibody (Cat No. SC-52, Santa Cruz biotech, US) diluted 1:800 in blocking solution at 4°C. Sections were washed 3 times in PBS for 10 minutes before incubation in biotinylated anti rabbit secondary antibody (1:600, Vectorlabs, Peterborough, UK) diluted in blocking solution for 2 hours at room temperature. Sections were washed 3 times in PBS for 10 minutes before incubation in ABC solution (ABC Kit, Vectorlabs, Peterborough, UK) for 1 hour at room temperature. Sections were washed 3 times in PBS for 10 minutes then incubated in DAB solution with Nickel Ammonium Sulfate (DAB kit, Vectorlabs, Peterborough, UK) for 5-10 minutes until precipitate developed. Sections were washed 3 times in PBS for 5 minutes to stop reaction.

Sections were mounted in PBS onto Superfrost plus slides (Thermofisher, US) and air-dried overnight. Sections were immersed in distilled water for 2 times 10 minutes then immediately dehydrated in an ethanol series immersed for in 30% followed by 60%, 90%, 95% and 100% ethanol for 2 minutes each then in clearing solution (Histoclear, National Diagnostics, US) for 10 minutes. Coverslips were applied using mounting medium (Histomount, National Diagnostics, US).

Images were captured at 10x magnification using a QI click camera (Qimaging) attached to an Olympus Bx53 microscope (Olympus). Sections at Bregma +1.18mm were selected for analysis. The number of cFos+ nuclei was quantified using ImageJ software (National Institutes of Health, MD, US). Sample areas of 250 by 750µm were specified within the NAc Core and Shell (Figure 5.3). Images were converted to binary image using an entropy based threshold (Kapur, Sahoo and Wong, 1985). Number of cFos+ nuclei was automatically counted within sample areas using the analyze particles function (specified objects of 50 to 100% circularity, 200-600 pixels).

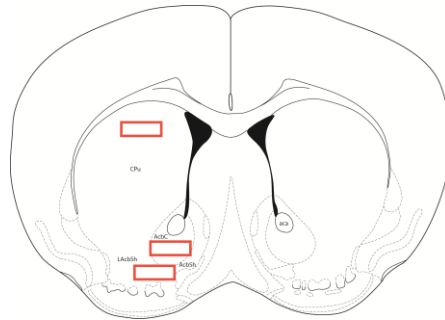


Figure 5.3. Schematic of regions sampled within Dorsal Striatum, NAc Core and NAc Shell indicated by red rectangles. Section at Bregma + 1.18mm.

5.2.9. Fluorescent in situ hybridisation (RNAScope)

5.2.9.1. Tissue Preparation

Mice were euthanised by intraperitoneal injection of sodium pentobarbital followed by cervical dislocation to minimise rupturing of blood vessels. Brains were extracted and flash-frozen by submergence in isopentane maintained at -50°C for 10 seconds then stored at -80°C prior to sectioning.

Brains were sectioned in an RNase free cryostat at -18°C . Brains were mounted on cryostat platforms using OCT mounting medium in -18°C chamber and left to equilibrate temperature for 1 hour prior to sectioning. Coronal sections of $10\mu\text{m}$ thickness were taken and mounted on SuperFrost Plus microscope slides (Thermofisher). Slides were stored at -80°C prior to in-situ-hybridisation.

Sections were submerged in 10% Buffered Formalin for 20 minutes at 4°C . Slides were washed in 1xPBS for 2 x 1 minute with gentle agitation then dehydrated in a series of ethanol solutions. Slides were submerged in 50% ethanol for 1 x 5 minutes, 70% ethanol 1 x 5 minutes and 100% ethanol for 2 x 5 minutes then incubated overnight in 100% ethanol at -20°C .

5.2.9.2. Procedure

In-situ-hybridisation was carried out using a manual RNAScope Fluorescent Multiplex Reagent Kit (Advanced Cell Diagnostics, Cat No. 320850) using instructions for fresh-frozen tissue and performing incubation steps using an ACD HybEZ™ Hybridization oven. We used RNAScope probes targeting: Mouse *cFos* (316928), *Drd1* (Cat No. 406491-C2) and *Drd2* (Cat No. 406501-C3). We

used the 'Amp4 Alt A' amplification reagent to label probes with fluorochromes as follows; *cFos* = Alexa-488, *Drd1* = Atto-647, and *Drd2* = Atto 550.

Images were captured using a QI click camera (Qimaging) attached to an Olympus Bx53 microscope (Olympus). Images of the NAc taken at 10x magnification were analyzed using ImageJ software (NIH).

Sample areas of 250 by 750µm were specified within the NAc Core and Shell (Figure 5.3). Images were converted to binary image using an entropy based threshold (Kapur, Sahoo and Wong, 1985). Cell nuclei stained by DAPI were used to select ROIs using the analyze particles function (specified objects of 50 to 100% circularity, 200-600 pixels). Pixel intensity for each probes signal was measured within each ROI representing a cell. Cells expressing *cFos* and either D1 or D2 were counted and compared in our analysis.

5.2.10. Statistical Analysis

All analyses were initially carried out using sex as a between subjects factor. Sex did not influence any outcomes and was therefore excluded from all analyses for clarity. All analyses were carried out using IBM SPSS software.

5.2.10.1. Behavioural Sensitisation to Cocaine

Behavioural sensitisation to cocaine was analysed using a mixed-factors ANOVA with genotype and treatment-drug as the between-subjects variables, session as the within-subject variable, and metres travelled in each session as the dependent variable. Following this treatment, behavioural sensitisation to cocaine was confirmed using a mixed-factors ANOVA with genotype and treatment-drug as the between-subjects variables, and difference in metres travelled between sessions 1 and 10 as the dependent variable.

To investigate whether any differences were present in baseline locomotor behaviour before the test sessions, an ANOVA was conducted with genotype as the between-subjects variable, day as the within-subject variable, and metres travelled during the habituation session as the dependent variable.

5.2.10.2. Conditioned Activity

Conditioned activity following behavioural sensitisation to cocaine was analysed using a mixed factors ANOVA, with genotype and drug dose as the between

subjects factors and metres travelled following a saline injection as the dependent variable.

5.2.10.3. Response to IP saline or cocaine (20mg/kg) challenge in sensitised vs non-sensitised animals

Response to 20mg/kg cocaine or saline challenge in non-sensitised/saline-treated mice was analysed using a mixed-factors ANOVA with genotype and challenge-drug as the between-subjects factors, and using metres travelled in the challenge session, and cFos expression in the NAc Core and Shell as the dependent variables.

Response to 20mg/kg cocaine or saline challenge in cocaine-sensitised mice was analysed using a mixed-factors ANOVA with genotype and challenge-drug as the between-subjects factors, and using metres travelled in the challenge session, and cFos expression in the NAc Core and Shell as the dependent variables.

Cocaine-induced responses in saline-treated and cocaine-sensitised mice were compared in a mixed factors ANOVA using treatment-drug and genotype as the between subjects factors, and using metres travelled in the challenge session, and cFos expression in the NAc Core and Shell as the dependent variables.

5.2.10.4. cFos Response to IP saline or cocaine (20mg/kg) challenge in sensitised vs non-sensitised animals in D1 vs D2 Neurons

Response to 20mg/kg cocaine or saline challenge in non-sensitised/saline-treated mice was analysed using a mixed-factors ANOVA with genotype and challenge-drug as the between-subjects factors, neuron-type as the within subjects variable, and using metres travelled in the challenge session and cFos expression, in D1 or D2 expressing neurons, in the NAc Core and Shell as the dependent variables.

Response to 20mg/kg cocaine or saline challenge in cocaine-sensitised mice was analysed using a mixed-factors ANOVA with genotype and challenge-drug as the between-subjects factors, using neuron-type as the within subjects variable, and using metres travelled in the challenge session, and cFos expression in the NAc Core and Shell the dependent variables.

Cocaine-induced responses in saline-treated and cocaine-sensitised mice were compared in a mixed factors ANOVA using treatment-drug and genotype as the between subjects factors, using neuron-type as the within subjects variable, and using metres travelled in the challenge session, and cFos expression in the NAC Core and Shell the dependent variables.

5.3. Results

5.3.1. Response to 20mg/kg acute cocaine challenge in $\alpha 4$ KO vs WT animals

Analysis of the habituation session (following IP saline) confirmed that there was no significant difference in baseline locomotor activity between $\alpha 4$ -WT ($n=8$) and $\alpha 4$ -KO ($n=8$) mice (non-significant effect of genotype $F_{(1,16)} = 0.02$, $p = 0.89$, NS). Compared with saline, an injection of 20mg/kg cocaine (IP) significantly increased locomotor activity, and there was no significant difference between $\alpha 4$ -WT ($n=4$) and $\alpha 4$ -KO ($n=4$) mice (Figure 5.4, significant main effect of drug $F_{(1,16)} = 77.74$, $p < 0.001$; non-significant effect of genotype $F_{(1,16)} = 0.424$, $p = 0.527$, NS; non-significant genotype by drug interaction $F_{(1,16)} = 1.84$, $p = 0.2$, NS).

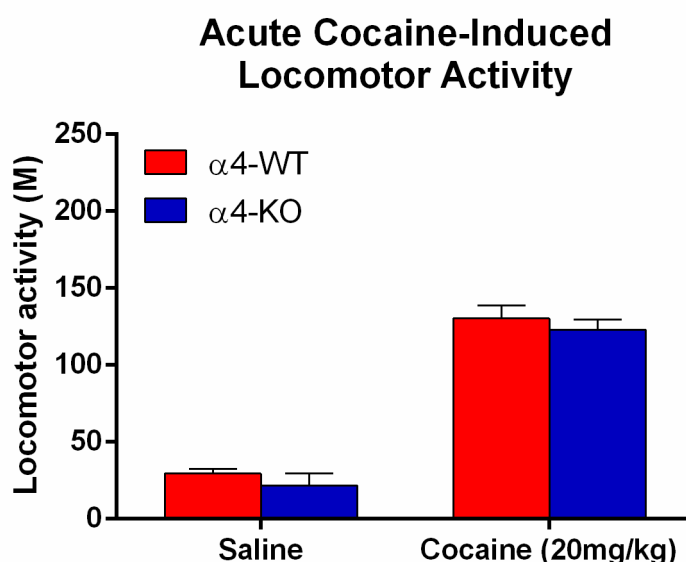


Figure 5.4. Effect of acute administration of saline or cocaine (20mg/kg, IP) injection on locomotor activity in $\alpha 4$ -WT ($n=8$) and $\alpha 4$ -KO ($n=8$) mice (per genotype; saline $n=4$, cocaine $n=4$). There was no significant difference in baseline or cocaine potentiated locomotor activity between genotypes. Error bars represent SEM.

5.3.2. cFos induced by acute cocaine (20mg/kg) in $\alpha 4$ -KO vs $\alpha 4$ -WT animals

Compared with saline, IP injection of cocaine (20mg/kg) significantly increased cFos expression in the NAc Core (Figure 5.5, significant main effect of drug $F_{(1,8)} = 82.15$, $p < 0.001$). Cocaine increased cFos to a greater extent in the NAc core of $\alpha 4$ -KO ($n=4$) than $\alpha 4$ -WT ($n=4$) mice (significant genotype by drug interaction $F_{(1,8)} = 7.563$, $p < 0.05$).

There was a significant main effect of genotype on cFos expression however this was driven by greater cocaine-induced cFos in $\alpha 4$ -KO mice (significant main-effect of genotype, $F_{(1,8)} = 9.48$, $p < 0.05$). Post hoc tests confirmed higher cFos expression in the NAc Core of cocaine challenged than saline challenged $\alpha 4$ -WT mice ($t_{(6)} = 3.91$, $p < 0.01$) and higher cFos expression in the NAc Core of cocaine challenged than saline challenged $\alpha 4$ -KO mice ($t_{(6)} = 4.1$, $p < 0.01$). Post hoc also confirmed that cFos expression in the NAc core was not significantly different in saline challenged $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice ($t_{(6)} = 0.327$, $p = 0.33$, NS) whereas, cocaine induced cFos was greater in $\alpha 4$ -KO ($n=4$) than $\alpha 4$ -WT ($n=4$) mice ($t_{(6)} = 30.4$, $p < 0.05$).

Cocaine challenge increased cFos in the NAc shell (Figure 5.5, significant main effect of drug $F_{(1,8)} = 47.66$, $p < 0.001$) and this was not significantly different in $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice (non-significant genotype by drug interaction $F_{(1,8)} = 0.055$, $p = 0.82$, NS). Overall cFos expression was not significantly different in the NAc shell of $\alpha 4$ -KO ($n=8$) and $\alpha 4$ -WT ($n=8$) mice (non-significant main effect of genotype, $F_{(1,8)} = 0.275$, $p = 0.61$, NS).

Cocaine challenge increased cFos in the Dorsal Striatum (Figure 5.5, significant main effect of drug $F_{(1,8)} = 97.95$, $p < 0.001$) and this was not significantly different in $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice (non-significant genotype by drug interaction $F_{(1,8)} = 0.18$, $p = 0.68$, NS). Overall cFos expression was not significantly different in the Dorsal Striatum of $\alpha 4$ -KO ($n=8$) and $\alpha 4$ -WT ($n=8$) mice (non-significant main effect of genotype, $F_{(1,8)} = 0.35$, $p = 0.56$, NS).

Acute Cocaine Induced cFos

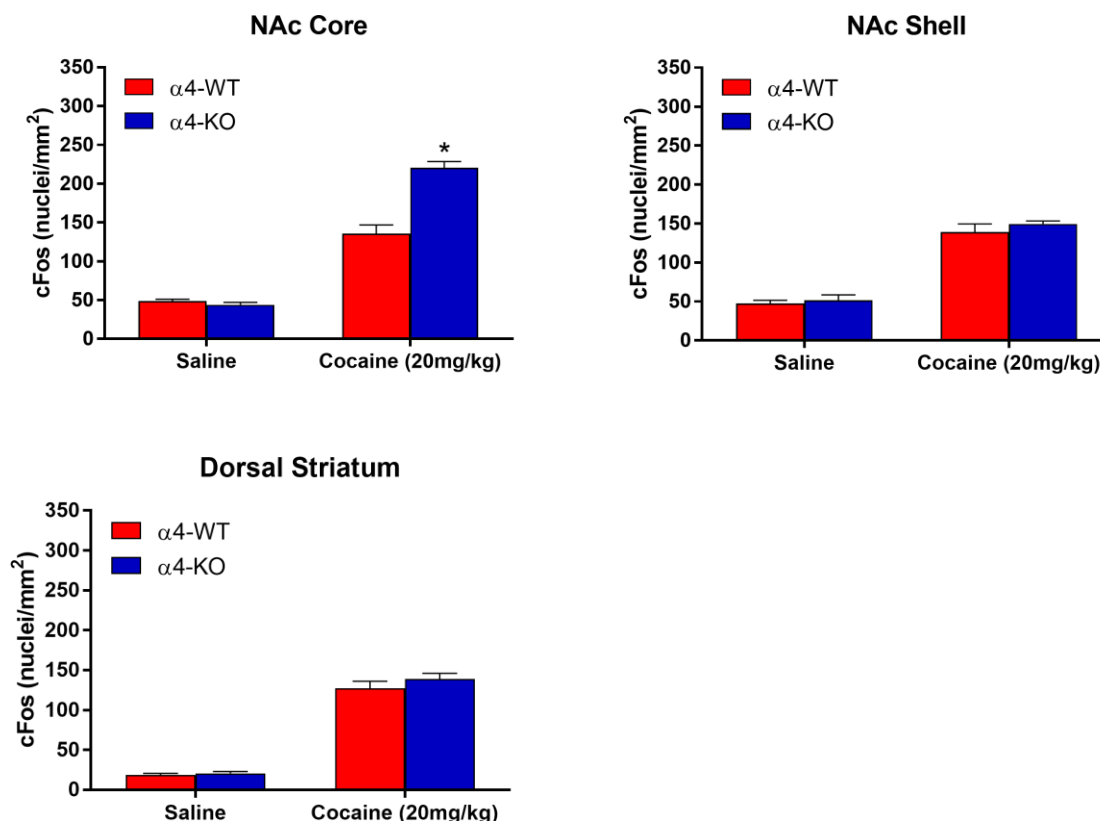


Figure 5.5. Effect of acute IP saline or cocaine injection on cFos expression the Dorsal striatum and Nucleus Accumbens of WT and $\alpha 4$ KO mice (per genotype; saline $n=4$, 20mg/kg cocaine $n=4$). Cocaine induced a greater level of cFos expression in the NAc Core ($p<0.001$) and did so to a greater extent in $\alpha 4$ KO mice ($p<0.05$). Cocaine induced a greater level of cFos in the NAc Shell ($p<0.001$) equally in both genotypes. Cocaine induced a greater level of cFos expression in the Dorsal Striatum ($p < 0.001$) similarly in both genotypes. Error bars represent SEM.

5.3.3. Behavioural Sensitisation to Cocaine (10mg/kg) in $\alpha 4$ -KO vs $\alpha 4$ -WT animals

Locomotor activity during habituation session (following IP saline) revealed no significant differences in baseline activity between genotypes (Figure 5.7, non-significant effect of genotype $F_{(1,8)} = 0.036$, $p = 0.855$, NS). Comparison of locomotor activity in session 1 revealed that injection of cocaine (10mg/kg)

increased locomotor activity similarly in both genotypes (Fig. non-significant effect of genotype $F_{(1,8)} = 0.24$, $p = 0.88$, NS).

Repeated, intermittent injections of cocaine (10mg/kg) induced an increase in locomotor activity over the course of 10 sessions (Figure 5.7, significant effect of session, $F_{(9,90)} = 5.98$, $p < 0.001$). There were no significant differences in activity between genotypes across the 10 sessions (Figure 5.7, non-significant main effect of genotype $F_{(1,10)} = 0.009$, $p = 0.93$, NS; non-significant session by genotype interaction $F_{(9,90)} = 0.006$, $p = 0.94$, NS).

Comparison of the difference between session 1 and session 10 activity confirmed that both genotypes sensitised to cocaine by a similar magnitude (non-significant effect of genotype, $F_{(1,8)} = 0.001$, $p = 0.97$, NS).

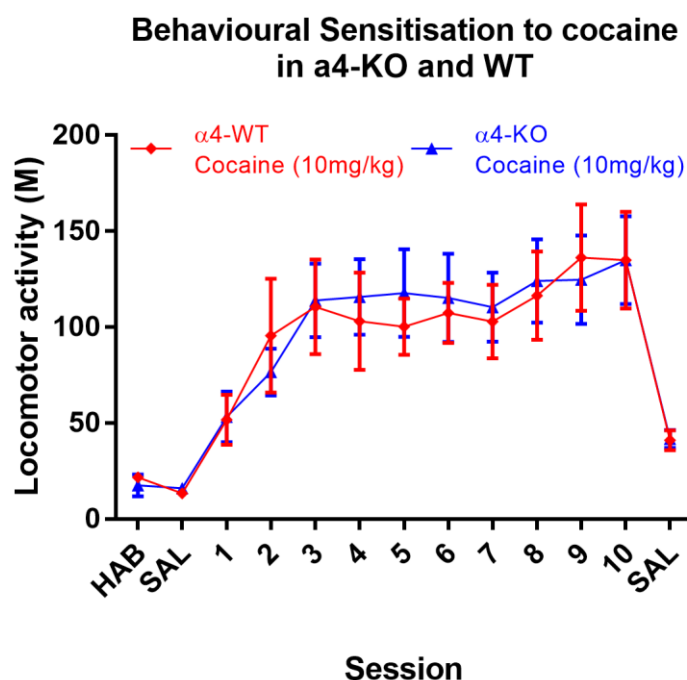


Figure 5.7. Effect of repeated intermittent cocaine on locomotor activity in $\alpha 4$ -WT ($n=8$) and $\alpha 4$ KO mice ($n=8$). Locomotor activity increased over the course of 10 sessions ($p<0.001$), equally in both genotypes. Error bars represent SEM.

5.3.4. Response to cocaine (20mg/kg) challenge in cocaine-sensitised $\alpha 4$ -KO and $\alpha 4$ -WT animals

Compared with saline, a challenge dose of cocaine (20mg/kg) significantly potentiated locomotor activity in cocaine-sensitised mice, and did so to a similar extent in both genotypes (Figure 5.8, significant effect of challenge-drug $F_{(1,8)} = 196.51$, $p < 0.001$; non-significant effect of genotype $F_{(1,8)} = 0.063$, $p = 0.81$, NS; non-significant genotype by challenge-drug interaction $F_{(1,8)} = 0.093$, $p = 0.77$).

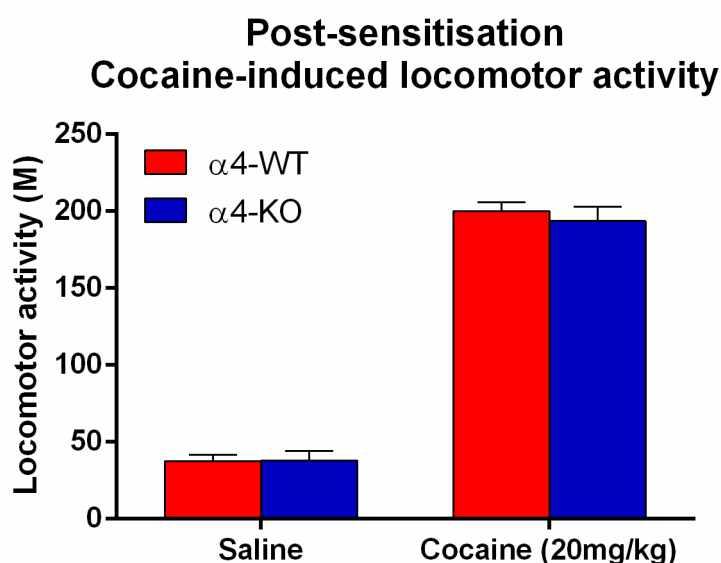


Figure 5.8. Effect of a challenge dose of 20mg/kg cocaine on locomotor activity in cocaine-sensitised WT and $\alpha 4$ KO mice (per genotype; saline $n=4$, cocaine $n=4$). Cocaine significantly increased locomotor activity ($p < 0.001$) equally in both genotypes. Error bars represent SEM.

5.3.5. cFos induced by cocaine (20mg/kg) in cocaine-sensitised $\alpha 4$ KO vs WT animals

Compared with saline, IP injection of cocaine (20mg/kg) significantly increased cFos expression in the NAc Core of cocaine-sensitised mice (Figure 5.9, significant main effect of drug $F_{(1,8)} = 48.03$, $p < 0.001$). Cocaine challenge increased cFos to a greater extent in the NAc Core of $\alpha 4$ -KO ($n=4$) than $\alpha 4$ -WT ($n=4$) cocaine-sensitised mice (Figure 5.9, significant genotype by drug interaction $F_{(1,8)} = 18.37$, $p < 0.01$).

There was a significant main effect of genotype (significant main effect of genotype, $F_{(1,8)} = 9.98$, $p < 0.05$) but this was driven by increased cocaine-induced cFos in $\alpha 4$ -KO mice. Post hoc tests confirmed higher cFos expression in the NAc Core of cocaine challenged than saline challenged $\alpha 4$ -WT mice ($t_{(6)} = 2.85$, $p < 0.05$) and higher cFos expression in the NAc Core of cocaine challenged than saline challenged $\alpha 4$ -KO mice ($t_{(6)} = 7.99$, $p < 0.001$). Post hoc tests also confirmed that cFos expression was similar in the NAc Core of saline challenged $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice ($t_{(6)} = 0.986$, $p = 0.38$, NS) whereas, cocaine induced cFos was greater in $\alpha 4$ -KO ($n=4$) than $\alpha 4$ -WT ($n=4$) mice ($t_{(6)} = 4.52$, $p < 0.05$).

Compared with saline, IP injection of cocaine (20mg/kg) significantly increased cFos expression in the NAc Shell of cocaine-sensitised mice (Figure 5.9, significant main effect of drug $F_{(1,8)} = 82.49$, $p < 0.001$). Cocaine challenge increased cFos to a greater extent in the NAc Shell of $\alpha 4$ -KO ($n=4$) than $\alpha 4$ -WT ($n=4$) mice (significant genotype by drug interaction $F_{(1,8)} = 20.4$, $p < 0.01$).

There was a significant main effect of genotype (significant main effect of genotype, $F_{(1,8)} = 0.275$, $p = 0.61$, NS), but this was driven by greater cFos expression in the NAc Shell of $\alpha 4$ -KO mice. Post hoc tests confirmed higher cFos expression in the NAc Shell of cocaine challenged than saline challenged $\alpha 4$ -WT mice ($t_{(6)} = 3.08$, $p < 0.01$) and higher cFos expression in the NAc Shell of cocaine challenged than saline challenged $\alpha 4$ -KO mice ($t_{(6)} = 10.15$, $p < 0.001$). Post hoc tests also confirmed that cFos expression was not significantly different in the NAc shell of saline challenged $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice ($t_{(6)} = 2.76$, $p = 0.17$, NS) whereas, cocaine induced cFos was greater in $\alpha 4$ -KO ($n=4$) than $\alpha 4$ -WT ($n=4$) mice ($t_{(6)} = 4.22$, $p < 0.05$).

Cocaine challenge increased cFos in the Dorsal Striatum in cocaine sensitised mice (Figure 5.9, significant main effect of drug $F_{(1,8)} = 84.54$, $p < 0.001$) and this was not significantly different in $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice (non-significant genotype by drug interaction $F_{(1,8)} = 0.18$, $p = 0.68$, NS). Overall cFos expression was not significantly different in the Dorsal Striatum of $\alpha 4$ -KO ($n=8$) and $\alpha 4$ -WT ($n=8$) mice (non-significant main effect of genotype, $F_{(1,8)} = 0.19$, $p = 0.68$, NS).

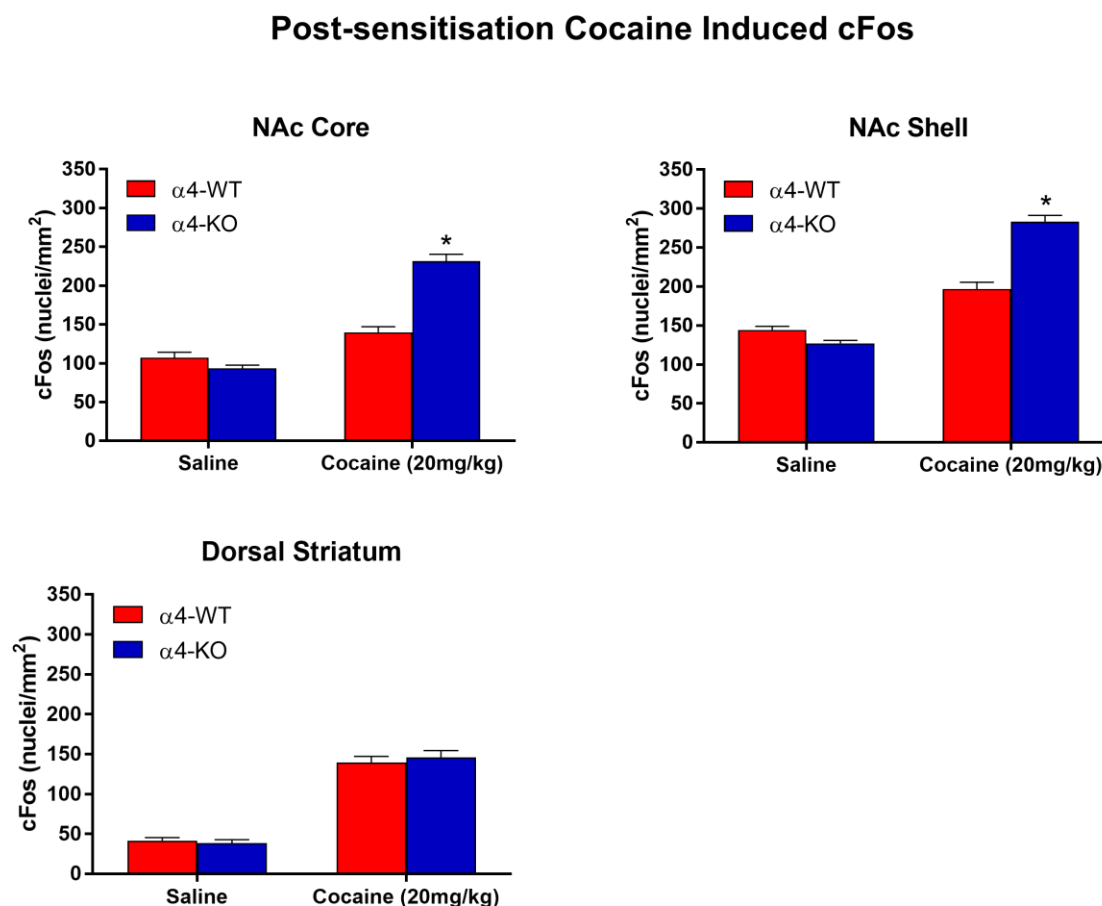


Figure 5.9. Effect of acute IP saline or cocaine injection on cFos expression the Dorsal Striatum and Nucleus Accumbens of WT and $\alpha 4$ KO mice (per genotype; saline $n=4$, 20mg/kg cocaine $n=4$). Compared with saline, cocaine induced a greater level of cFos expression in the NAc Core ($p<0.01$) and did so to a greater extent in $\alpha 4$ KO mice ($p<0.05$). Cocaine induced a greater level of cFos in the NAc Shell ($p<0.001$) and did so to a greater extent in $\alpha 4$ KO mice ($p<0.01$). Cocaine induced a greater level of cFos expression in the Dorsal Striatum ($p < 0.001$) similarly in both genotypes. Error bars represent SEM.

5.3.6. Behavioural Sensitisation to Cocaine in D1- $\alpha 4$ -KO vs D1- $\alpha 4$ -WT animals

Locomotor activity during the initial habituation session (following IP saline injection) revealed no significant differences in baseline locomotor activity between $\alpha 4$ -D1-WT ($n=16$) and $\alpha 4$ -D1-KO ($n=16$) mice (Figure 5.11, non-significant effect of genotype $F_{(1,28)} = 0.847$, $p = 0.365$, NS).

Comparison of activity in session 1 revealed that, relative to saline, acute-cocaine injection (10mg/kg) significantly increased locomotor activity (Figure 5.11, significant main effect of treatment-drug $F_{(1,28)} = 46.89$, $p < 0.001$) and did so to a greater extent in $\alpha 4$ -D1-KO ($n=8$) than $\alpha 4$ -D1-WT ($n=8$) (Figure 5.11, significant genotype by treatment-drug interaction $F_{(1,28)} = 4.19$, $p < 0.05$).

Repeated, intermittent injections of cocaine, but not saline, induced an increase in locomotor activity over the course of 10 sessions (Figure 5.11, significant main effect of treatment-drug, $F_{(1,28)} = 199.097$, $p < 0.001$; significant session by treatment-drug interaction, $F_{(9,252)} = 11.231$, $p < 0.001$). Saline-treated $\alpha 4$ -D1-WT and $\alpha 4$ -D1-KO mice showed a similar locomotor activity over 10 sessions (non-significant session by genotype interaction $F_{(9,252)} = 0.405$, $p = 0.53$, NS). There were significant differences in cocaine-induced activity between genotypes, whereby $\alpha 4$ -D1-KO mice showed enhanced cocaine induced locomotor activity over the first 3 sessions (Figure 5.11, significant session by genotype by treatment-drug interaction, $F_{(9,252)} = 4.13$, $p < 0.05$).

To compare cocaine-sensitisation between genotypes we measured the difference in activity between session 1 and session 10. Comparison of this difference confirmed that both genotypes sensitised to cocaine by a similar magnitude (significant effect of treatment-drug, $F_{(1,28)} = 24.12$, $p < 0.001$, non-significant treatment-drug by genotype interaction, $F_{(1,28)} = 3.34$, $p = 0.079$, NS).

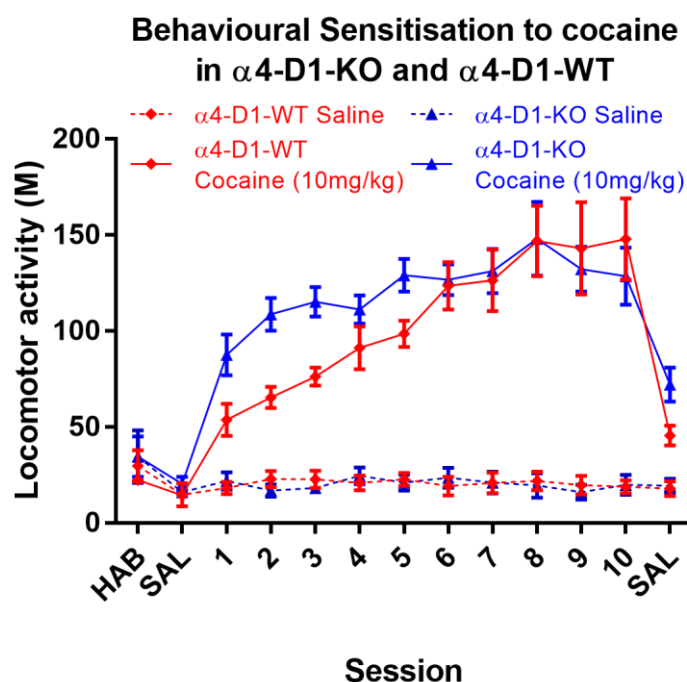


Figure 5.11. Effect of repeated intermittent cocaine on locomotor activity in D1- $\alpha 4$ -D1-WT and $\alpha 4$ -D1-KO mice (per genotype: saline $n=8$, cocaine $n=8$). Locomotor activity was increased over the course of 10 sessions of cocaine administration ($p<0.001$) however in D1- $\alpha 4$ -KO mice exhibited more rapid sensitisation ($p<0.05$). In session 1 acute cocaine injection increased locomotor activity ($p<0.001$), to a greater extent in D1- $\alpha 4$ -KO mice ($p<0.001$). Error bars represent SEM.

5.3.7. Conditioned Activity in D1- $\alpha 4$ -KO vs D1- $\alpha 4$ -WT animals

Following 10 days of repeated cocaine, but not saline, mice showed conditioned increases in activity following a saline injection in the cocaine-paired environment (Fig; significant main effect of treatment-drug, $F_{(1,28)} = 48.72$, $p < 0.001$). Conditioned activity was significantly greater in $\alpha 4$ -D1-KO ($n=8$) compared to $\alpha 4$ -D1-WT ($n=8$) mice (Figure 5.12, significant genotype by treatment-drug interaction $F_{(1,28)} = 4.77$, $p<0.05$).

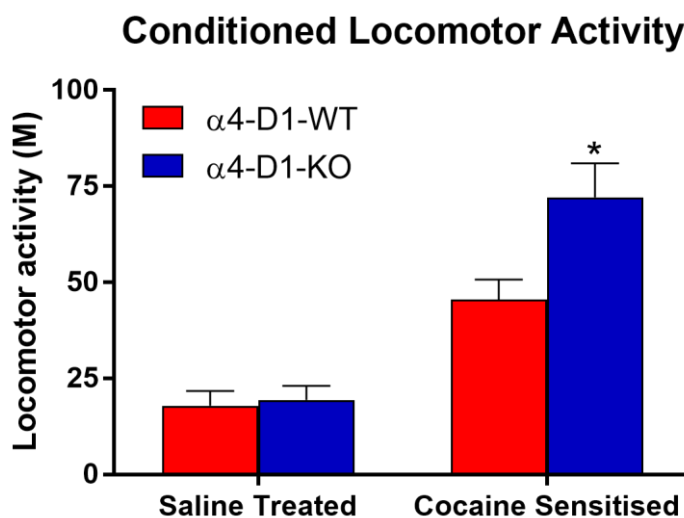


Figure 5.12. Conditioned activity in cocaine-sensitised or saline-treated α4-D1-WT and α4-D1-KO mice (n=8 per group). Locomotor activity following saline administration was significantly increased in cocaine-sensitised mice compared with saline-treated mice ($p < 0.001$). α4-D1-KO mice showed significantly greater conditioned activity ($p < 0.05^*$). Error bars represent SEM.

5.3.8. Response to 20mg/kg cocaine challenge in sensitised and non-sensitised D1-α4-KO vs D1-α4-WT animals

Compared with saline, cocaine challenge (20mg/kg) significantly increased locomotor activity in saline-treated mice (significant main effect of challenge-drug $F_{(1,16)} = 74.9$, $p < 0.001$), to a greater extent in saline-treated α4-D1-KO (n=4) than α4-D1-WT (n=4) mice (Figure 5.13, significant challenge-drug by genotype interaction, $F_{(1,16)} = 9.8$, $p < 0.01$).

Compared with saline, cocaine challenge (20mg/kg) significantly increased locomotor activity in cocaine-sensitised mice (Figure 5.13, significant main effect of challenge-drug $F_{(1,16)} = 120.95$, $p < 0.001$), and this was not significantly different between cocaine-sensitised α4-D1-KO (n=4) and α4-D1-WT (n=4) mice (non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 1.25$, $p = 0.29$, NS).

Cocaine-induced locomotor activity was significantly greater in cocaine-sensitised than non-sensitised mice (Figure 5.13, significant main effect of treatment-drug $F_{(1,16)} = 18.81$, $p < 0.001$, NS) and this was not significantly

different between $\alpha 4$ -WT ($n=4$) and $\alpha 4$ -KO ($n=4$) mice (non-significant treatment-drug by genotype interaction $F_{(1,16)} = 0.18$, $p = 0.68$, NS).

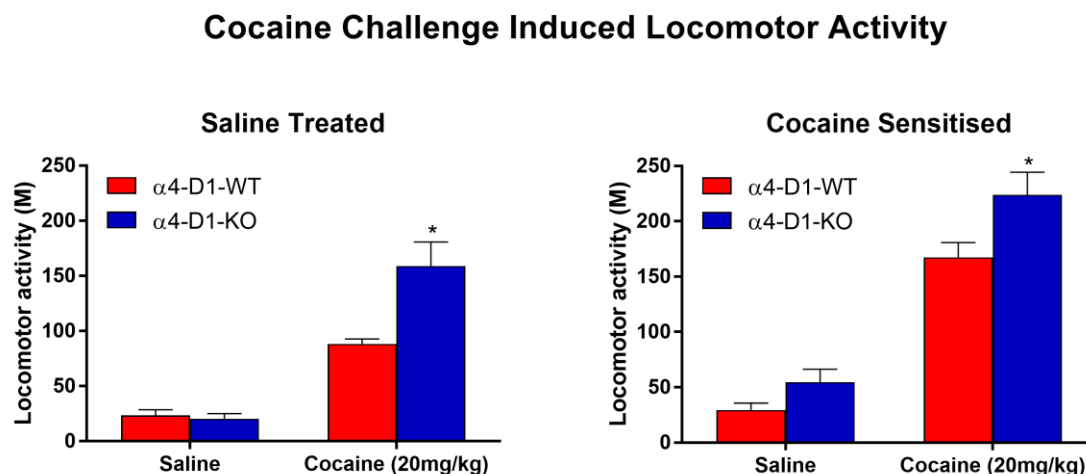


Figure 5.13. Effect of challenge dose of 20mg/kg cocaine on saline-treated and cocaine-sensitised $\alpha 4$ -D1-WT vs $\alpha 4$ -D1-KO mice ($n=8$ per group). Cocaine significantly increased locomotor activity ($p<0.001$), to a greater extent in D1- $\alpha 4$ -KO mice ($p<0.001$). Cocaine-sensitisation increased response to the cocaine challenge ($p<0.001$) similarly in both genotypes. Error bars represent SEM.

5.3.9. cFos induced by cocaine in sensitised and non-sensitised $\alpha 4$ -D1-KO vs $\alpha 4$ -D1-WT animals

To analyse the effects of acute cocaine in sensitised and non-sensitised animals we compared cFos expression in the Dorsal Striatum and Nucleus Accumbens following an acute challenge of either saline or cocaine (20mg/kg) in cocaine-sensitised vs saline-treated animals.

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc Core of saline-treated mice (Figure 5.14, significant main effect of challenge-drug $F_{(1,16)} = 51.66$, $p<0.001$), to a greater extent in $\alpha 4$ -D1-KO ($n=4$) than $\alpha 4$ -D1-WT ($n=4$) mice (Figure 5.14, significant challenge-drug by genotype interaction, $F_{(1,16)} = 11.91$, $p<0.01$).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc Core of cocaine-sensitised mice (Figure 5.14, significant main effect of challenge-drug $F_{(1,16)} = 25.29$, $p<0.001$), to a greater extent in

$\alpha 4$ -D1-KO (n=4) than $\alpha 4$ -D1-WT (n=4) mice (Figure 5.14, significant challenge-drug by genotype interaction, $F_{(1,16)} = 6.77$, $p < 0.05$, non-significant main effect of genotype $F = 2.52$, $p = 0.138$, NS).

Cocaine-induced cFos in the NAc core was significantly greater in cocaine-sensitised than saline-treated mice (Figure 5.14, significant main effect of treatment-drug $F_{(1,16)} = 12.37$, $p < 0.001$, NS) and this was not significantly different between $\alpha 4$ -D1-WT (n=4) and $\alpha 4$ -D1-KO (n=4) mice (Figure 5.14, non-significant treatment-drug by genotype interaction $F_{(1,16)} = 0.288$, $p = 0.6$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc shell of saline-treated mice (Figure 5.14, significant main effect of challenge-drug $F_{(1,16)} = 177.3$, $p < 0.001$), and this was not significantly different between $\alpha 4$ -D1-KO (n=4) and $\alpha 4$ -D1-WT (n=4) mice (Figure 5.14, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.14$, $p = 0.71$, NS, non-significant main effect of genotype $F_{(1,16)} = 1.08$, $p = 0.319$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc shell of cocaine-sensitised mice (Figure 5.14, significant main effect of challenge-drug $F_{(1,16)} = 67.55$, $p < 0.001$), to a greater extent in $\alpha 4$ -D1-KO (n=4) than $\alpha 4$ -D1-WT (n=4) mice (Figure 5.14, significant challenge-drug by genotype interaction, $F_{(1,16)} = 4.96$, $p < 0.05$).

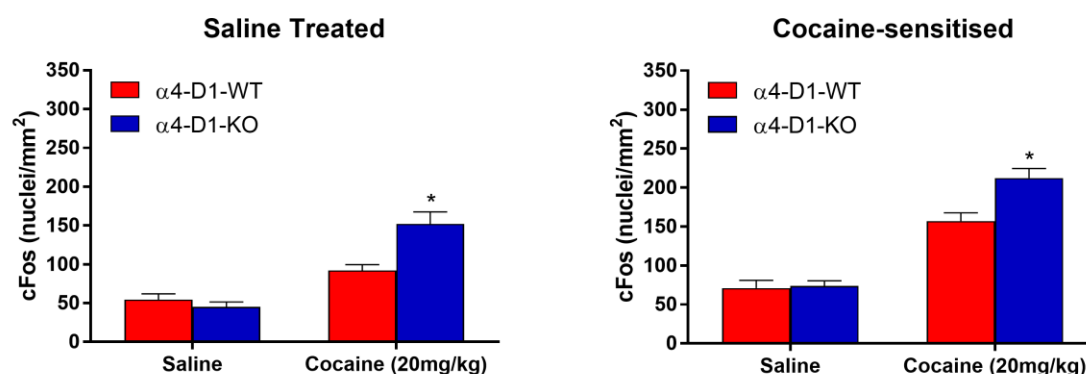
Cocaine-induced cFos in the NAc shell was significantly greater in cocaine-sensitised than saline-treated mice (Figure 5.14, significant main effect of treatment-drug $F_{(1,16)} = 36.34$, $p < 0.001$, NS) and this was not significantly different between $\alpha 4$ -D1-WT (n=4) and $\alpha 4$ -D1-KO (n=4) mice (Figure 5.14, non-significant treatment-drug by genotype interaction $F_{(1,16)} = 3.38$, $p = 0.091$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the Dorsal Striatum of saline-treated mice (Figure 5.14, significant main effect of challenge-drug $F_{(1,16)} = 154.03$, $p < 0.001$), and this was not significantly different between $\alpha 4$ -D1-KO (n=4) and $\alpha 4$ -D1-WT (n=4) mice (Figure 5.14, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.15$, $p = 0.71$, NS, non-significant main effect of genotype $F_{(1,16)} = 0.041$, $p = 0.843$, NS).

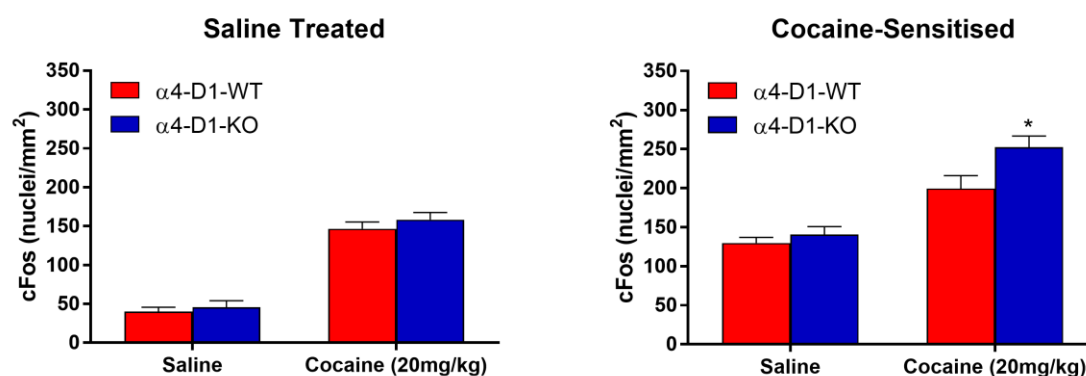
Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the Dorsal Striatum of cocaine-sensitised mice (Figure 5.14, significant main effect of challenge-drug $F_{(1,16)} = 68.15$, $p < 0.001$), and this was not significantly different between $\alpha 4$ -D1-KO ($n=4$) and $\alpha 4$ -D1-WT ($n=4$) mice (Figure 5.14, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.086$, $p = 0.774$, NS).

Cocaine-induced cFos in the Dorsal Striatum was significantly greater in cocaine-sensitised than saline-treated mice (Figure 5.14, significant main effect of treatment-drug $F_{(1,16)} = 4.83$, $p < 0.05$) and this was not significantly different between $\alpha 4$ -D1-WT ($n=4$) and $\alpha 4$ -D1-KO ($n=4$) mice (Figure 5.14, non-significant treatment-drug by genotype interaction $F_{(1,16)} = 0.16$, $p = 0.69$, NS).

Cocaine-induced cFos in NAc Core



Cocaine-induced cFos in NAc Shell



Cocaine Induced cFos in Dorsal Striatum

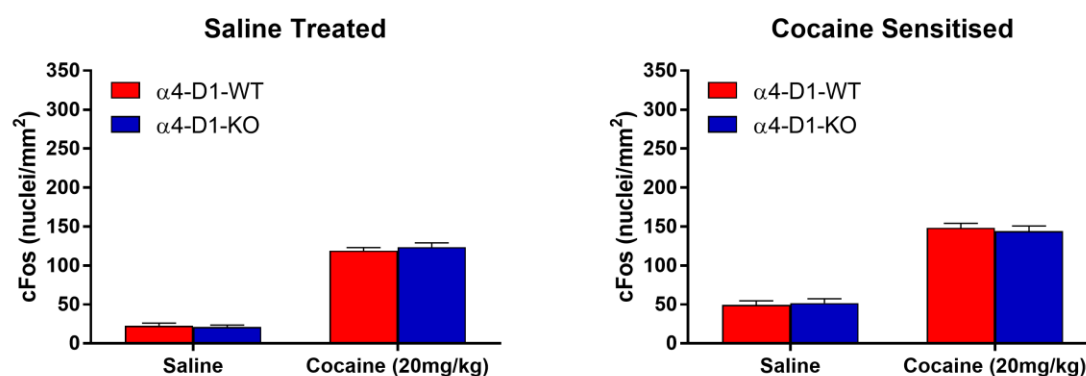
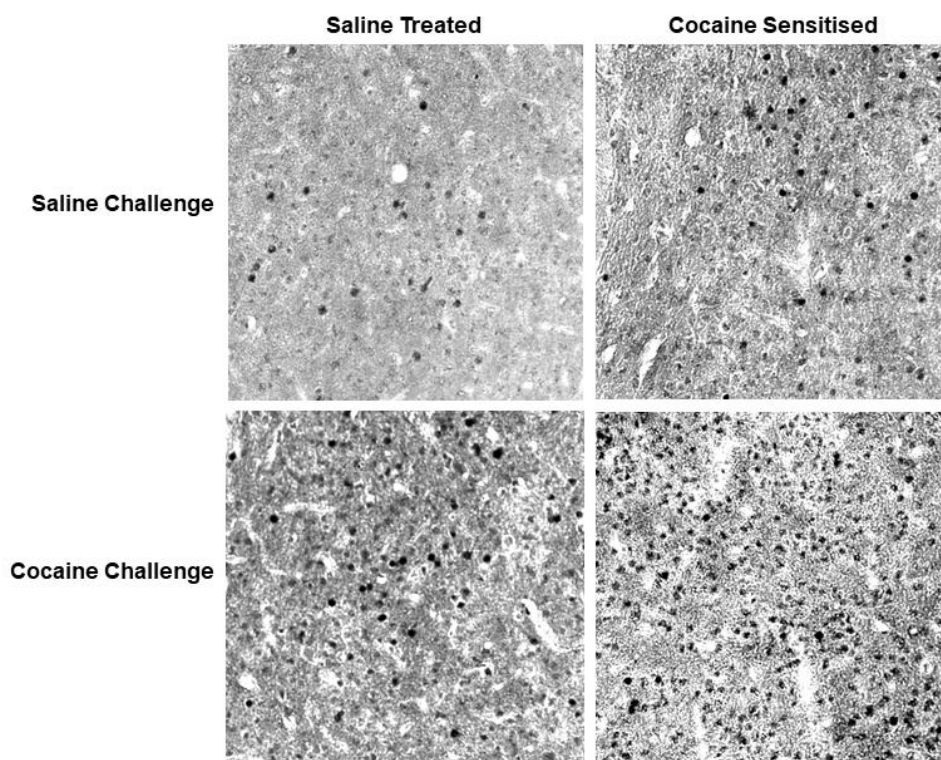
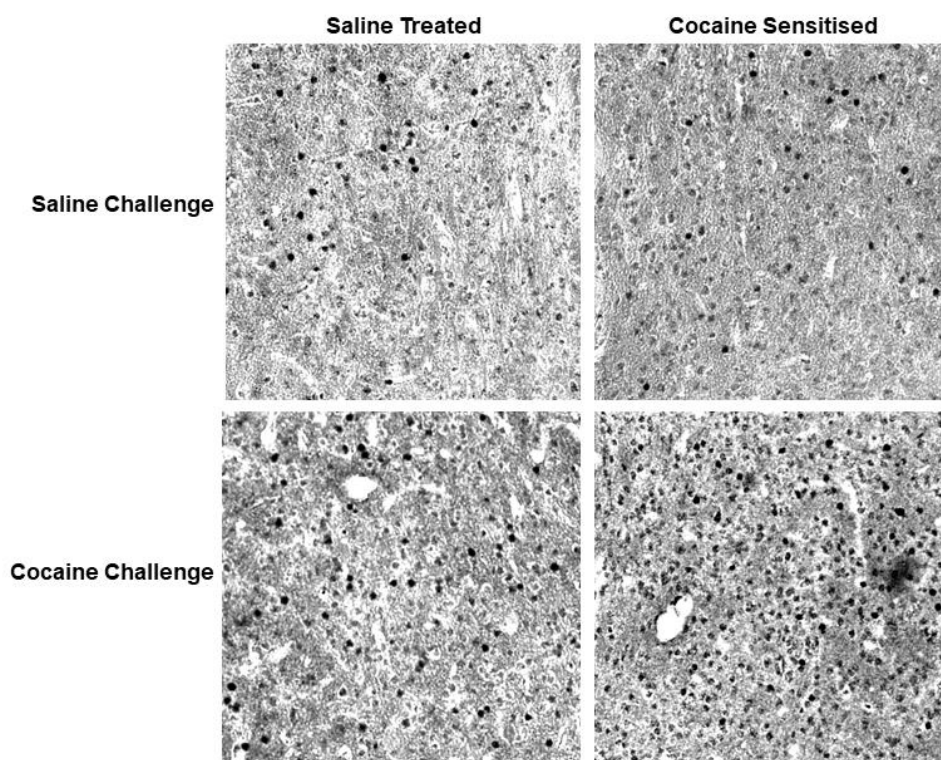


Figure 5.14. Effect of IP saline or cocaine (20mg/kg) injection on cFos expression the NAc Core and Shell of cocaine-sensitised or saline-treated $\alpha 4$ -D1-WT and $\alpha 4$ -D1-KO mice (n=8 per group). In saline-treated mice acute cocaine challenge increased cFos expression in the NAc Core ($p < 0.001$), to a greater

extent in $\alpha 4$ -D1-KO mice ($p < 0.01^*$). In saline-treated mice acute cocaine challenge increased cFos expression in the NAc Shell ($p < 0.001$) and this was not different between genotypes. In cocaine-sensitised mice cocaine challenge increased cFos expression in the NAc Core ($p < 0.001$), to a greater extent in $\alpha 4$ -D1-KO mice ($p < 0.05^*$). In cocaine-sensitised mice acute cocaine challenge increased cFos expression in the NAc Shell ($p < 0.001$) to a greater extent in $\alpha 4$ -D1-KO mice ($p < 0.05^*$). Cocaine challenge induced greater cFos expression in the NAc Core of cocaine-sensitised than saline-treated mice ($p < 0.001$) and this was not different between genotypes. Cocaine challenge induced greater cFos expression in the NAc shell of cocaine-sensitised than saline-treated mice ($p < 0.001$) and this was not different between genotypes. Cocaine challenge induced greater cFos expression in the Dorsal Striatum of saline-treated ($p < 0.001$) and cocaine-sensitised mice ($p < 0.001$) and this was not different between genotypes. Error bars represent SEM.

**α 4-D1-WT
NAc Core** **α 4-D1-KO
NAc Core**

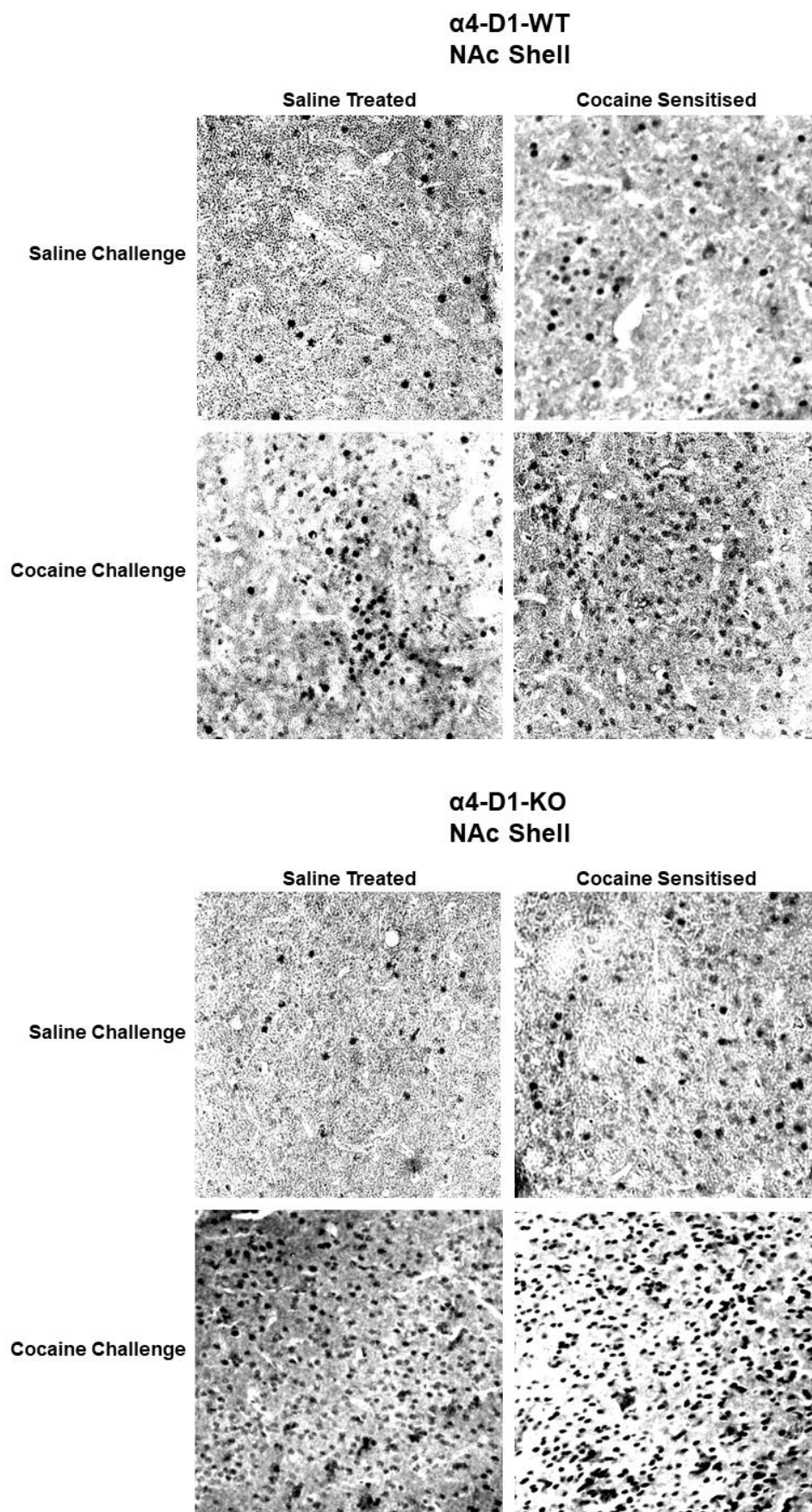


Figure 5.15. Representative images NAc Core and Shell following cocaine (20mg/kg) and saline challenge in cocaine-sensitised and saline-treated α 4-D1-WT and α 4-D1-KO mice.

5.3.10. Behavioural Sensitisation to Cocaine in $\alpha 4$ -D2-KO vs $\alpha 4$ -D2-WT animals

Locomotor activity during the initial habituation session (following IP saline injection) revealed no significant differences in baseline locomotor activity between $\alpha 4$ -D2-WT ($n=16$) and $\alpha 4$ -D2-KO ($n=16$) mice (Figure 5.16, non-significant effect of genotype $F_{(1,28)} = 0.91$, $p = 0.348$, NS).

Comparison of activity in session 1 revealed that, relative to saline, acute-cocaine injection (10mg/kg) significantly increased locomotor activity (Figure 5.16, significant main effect of treatment-drug $F_{(1,28)} = 35.02$, $p < 0.001$), to a similar extent in D2- $\alpha 4$ -KO ($n=8$) and D2- $\alpha 4$ -WT ($n=8$) mice (Figure 5.16, non-significant genotype by treatment-drug interaction $F_{(1,28)} = 0.365$, $p = 0.55$, NS).

Repeated, intermittent injections of cocaine, but not saline, induced an increase in locomotor activity over the course of 10 sessions (Figure 5.16, significant main effect of treatment-drug, $F_{(1,28)} = 32.7$, $p < 0.001$; significant session by treatment-drug interaction, $F_{(9,252)} = 8.6$, $p < 0.01$). Saline-treated $\alpha 4$ -D2-WT and $\alpha 4$ -D2-KO mice showed a similar locomotor activity over 10 sessions (Figure 5.16, non-significant session by genotype interaction $F_{(9,25)} = 2.89$, $p = 0.329$, NS). Cocaine-induced activity was similar in both genotypes (Figure 5.16, non-significant session by genotype by treatment-drug interaction, $F_{(9,124)} = 1.57$, $p = 0.22$, NS).

To compare cocaine-sensitisation between genotypes we measured the difference in activity between session 1 and session 10. Comparison of this difference confirmed that both genotypes sensitised to cocaine by a similar magnitude (Figure 5.16, significant effect of treatment-drug, $F_{(1,28)} = 8.57$, $p < 0.01$, non-significant treatment-drug by genotype interaction, $F_{(1,28)} = 0.05$, $p = 0.819$, NS).

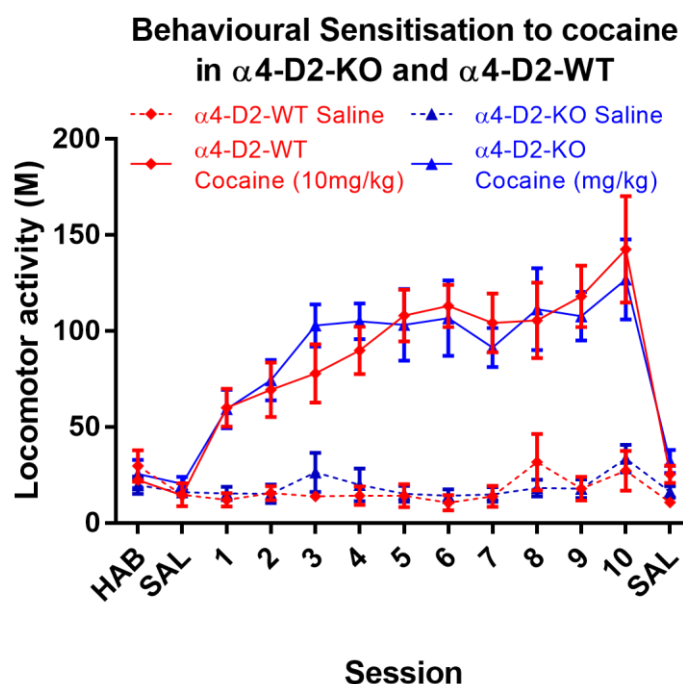


Figure 5.16. Effect of repeated intermittent cocaine on locomotor activity in $\alpha 4$ -D2-WT and $\alpha 4$ -D2-KO mice (per genotype; saline $n=8$, cocaine $n=8$). Locomotor activity was increased over the course of 10 sessions of cocaine administration ($p<0.001$) similarly in both genotypes. In session 1 acute cocaine injection increased locomotor activity similarly in both genotypes. Error bars represent SEM.

5.3.11. Conditioned Activity in $\alpha 4$ -D2-KO vs $\alpha 4$ -D2-WT animals

Following 10 days of repeated cocaine, but not saline, mice showed increases in activity following a saline injection in the cocaine-paired environment (Figure 5.17, significant main effect of treatment-drug, $F_{(1,28)} = 12.98$, $p < 0.001$). Conditioned activity was similar in $\alpha 4$ -D2-KO ($n=8$) and $\alpha 4$ -D2-WT ($n=8$) mice (non-significant genotype by treatment-drug interaction $F_{(1,28)} = 0.199$, $p = 0.659$).

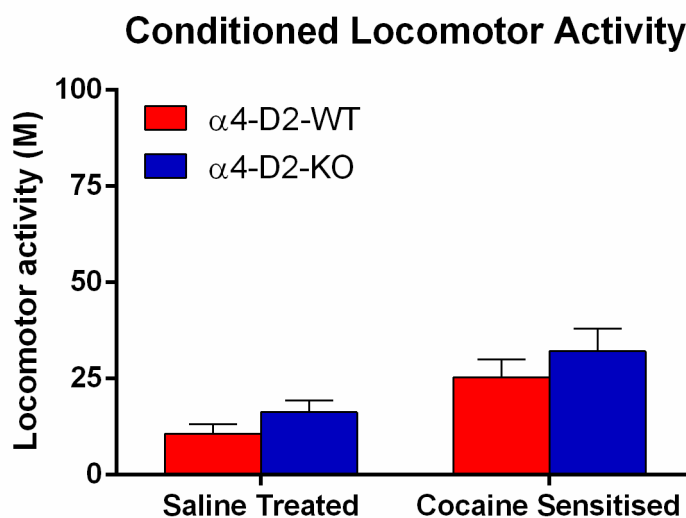


Figure 5.17. Conditioned activity in cocaine-sensitised or saline-treated α4-D2-WT and α4-D2-KO mice (n=8 per group). Locomotor activity following saline administration was significantly increased in cocaine-sensitised mice than saline-treated mice ($p < 0.001$), and this was not different between genotypes. Error bars represent SEM.

5.3.12. Response to 20mg/kg cocaine challenge in sensitised and non-sensitised α4-D2-KO vs α4-D2-WT animals

Compared with saline, cocaine challenge (20mg/kg) significantly increased locomotor activity in saline-treated mice (Figure 5.18, significant main effect of challenge-drug $F_{(1,16)} = 189.01$, $p < 0.001$), and this was not significantly different between in α4-D2-KO (n=4) and α4-D2-WT (n=4) mice (Figure 5.18, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 2.868$, $p = 0.116$, NS; non-significant main effect of genotype, $F_{(1,16)} = 1.28$, $p = 0.281$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased locomotor activity in cocaine-sensitised mice (Figure 5.18, significant main effect of challenge-drug $F_{(1,16)} = 123.84$, $p < 0.001$), and this was not significantly different between in α4-D2-KO (n=4) and α4-D2-WT (n=4) mice (Figure 5.18, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.002$, $p = 0.968$, NS; non-significant main effect of genotype, $F_{(1,16)} = 0.12$, $p = 0.73$, NS).

Cocaine-induced locomotor activity was significantly greater in cocaine-sensitised than non-sensitised mice (Figure 5.18, significant main effect of treatment-drug $F_{(1,16)} = 18.81$, $p < 0.001$, NS) and this was not significantly different between $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (Figure 5.18, non-significant treatment-drug by genotype interaction $F_{(1,16)} = 0.18$, $p = 0.68$, NS).

Cocaine Challenge Induced Locomotor Activity

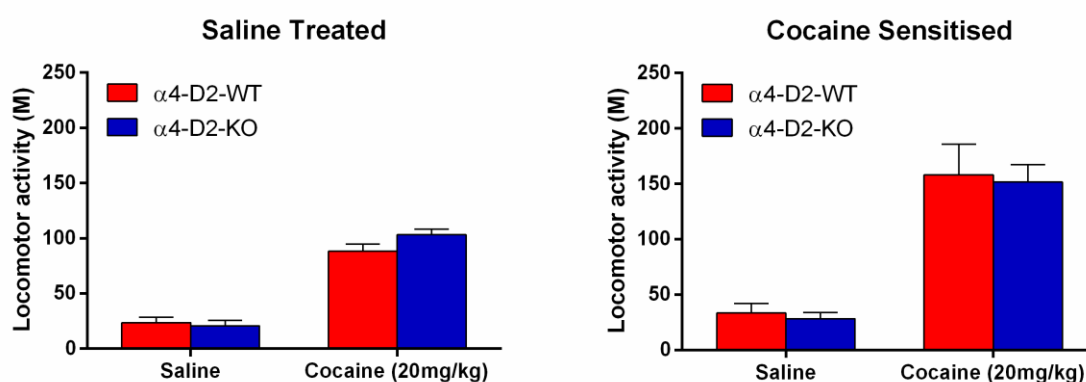


Figure 5.18. Effect of challenge dose of 20mg/kg cocaine on saline-treated and cocaine-sensitised $\alpha 4$ -D2-WT and $\alpha 4$ -D2-KO mice ($n=8$ per group). Cocaine significantly increased locomotor activity ($p<0.001$) to a greater extent in cocaine-sensitised mice than saline-treated mice ($p<0.001$) and this was not different between. Error bars represent SEM.

5.3.13. cFos induced by cocaine in sensitised and non-sensitised D2- $\alpha 4$ -KO vs D2- $\alpha 4$ -WT animals

To analyse the effects of acute cocaine in sensitised and non-sensitised animals we compared cFos expression in the NAc Core and Shell following an acute challenge of either saline or cocaine (20mg/kg) in cocaine-sensitised vs saline-treated animals.

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc Core of saline-treated mice (Figure 5.19, significant main effect of challenge-drug $F_{(1,16)} = 35.5$, $p<0.001$), and this was not significantly

different between in $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (Figure 5.19, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 1.57$, $p = 0.23$, NS; non-significant main effect of genotype $F = 0.04$, $p = 0.84$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc Core of cocaine-sensitised mice (Figure 5.19, significant main effect of challenge-drug $F_{(1,16)} = 45.24$, $p < 0.001$), and this was not significantly different between in $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (Figure 5.19, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 2.71$, $p = 0.13$, NS; non-significant main effect of genotype $F_{(1,16)} = 0.086$, $p = 0.78$, NS).

Cocaine-induced cFos in the NAc core was significantly greater in cocaine-sensitised than saline-treated mice (Figure 5.19, significant main effect of treatment-drug $F_{(1,16)} = 11.31$, $p < 0.01$) and this was not significantly different between in $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (Figure 5.19, non-significant treatment-drug by genotype interaction $F_{(1,16)} = 3.26$, $p = 0.096$, NS; non-significant main effect of genotype $F_{(1,16)} = 0.16$, $p = 0.7$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc shell of saline-treated mice (significant main effect of challenge-drug $F_{(1,16)} = 59.4$, $p < 0.001$), and this was not significantly different between in $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (Figure 5.19, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.033$, $p = 0.86$, NS; non-significant main effect of genotype $F_{(1,16)} = 0.38$, $p = 0.55$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc shell of cocaine-sensitised mice (Figure 5.19, significant main effect of challenge-drug $F_{(1,16)} = 4.65$, $p < 0.05$), and this was not significantly different between in $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.11$, $p = 0.75$, NS; non-significant main effect of genotype $F_{(1,16)} = 0.17$, $p = 0.69$, NS).

Cocaine-induced cFos in the NAc shell was significantly greater in cocaine-sensitised than saline-treated mice (Figure 5.19, significant main effect of treatment-drug $F_{(1,16)} = 10.58$, $p < 0.01$) and this was not significantly different between in $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (Figure 5.19, non-

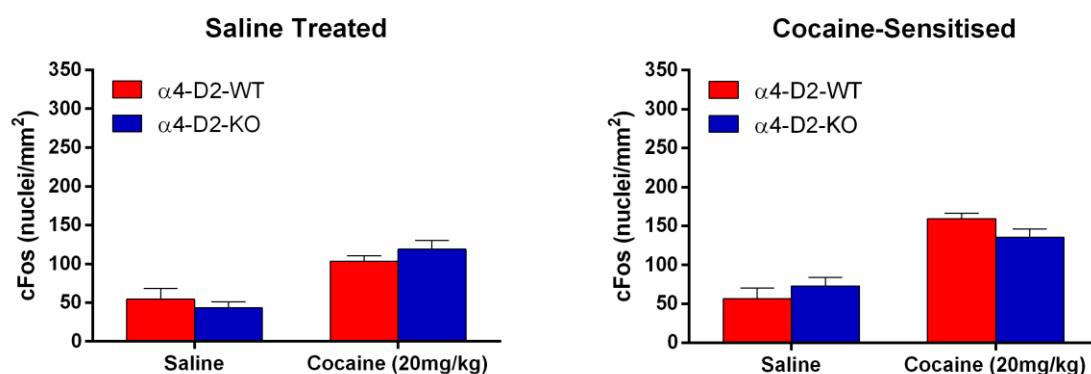
significant treatment-drug by genotype interaction $F_{(1,16)} = 0.056$, $p = 0.82$, NS; non-significant main effect of genotype $F_{(1,16)} = 0.022$, $p = 0.86$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the Dorsal Striatum of saline-treated mice (Figure 5.19, significant main effect of challenge-drug $F_{(1,16)} = 91.95$, $p < 0.001$), and this was not significantly different between $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (Figure 5.19, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.021$, $p = 0.89$, NS; non-significant main effect of genotype $F_{(1,16)} = 0.14$, $p = 0.715$, NS).

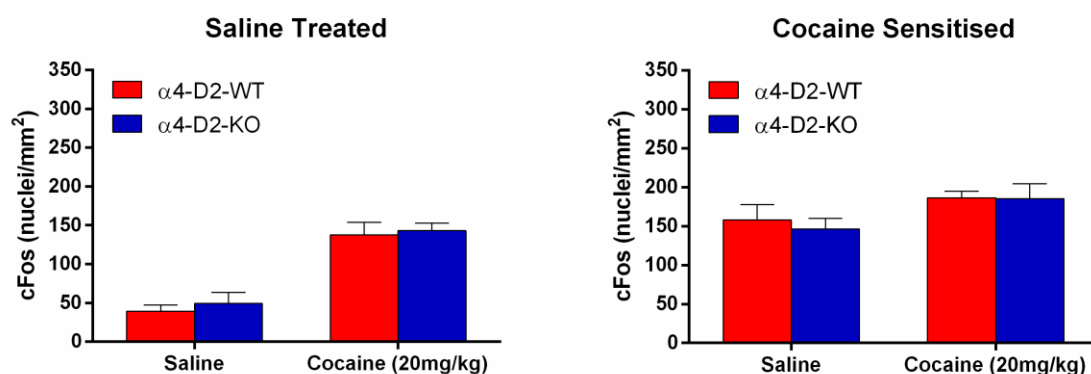
Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the Dorsal Striatum of cocaine-sensitised mice (Figure 5.14, significant main effect of challenge-drug $F_{(1,16)} = 101.41$, $p < 0.001$), and this was not significantly different between $\alpha 4$ -D2-KO ($n=4$) and $\alpha 4$ -D2-WT ($n=4$) mice (Figure 5.19, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.686$, $p = 0.424$, NS).

Cocaine-induced cFos in the Dorsal Striatum was not-significantly different between cocaine-sensitised than saline-treated mice, although there was a trend towards increased cFos in cocaine-induced cFos in cocaine-sensitised mice (Figure 5.14, significant main effect of treatment-drug $F_{(1,16)} = 4.07$, $p = 0.067$) and this was not significantly different between $\alpha 4$ -D2-WT ($n=4$) and $\alpha 4$ -D2-KO ($n=4$) mice (Figure 5.14, non-significant treatment-drug by genotype interaction $F_{(1,16)} = 0.117$, $p = 0.738$, NS).

Cocaine-induced cFos in NAc Core



Cocaine-induced cFos in NAc Shell



Cocaine-induced cFos in Dorsal Striatum

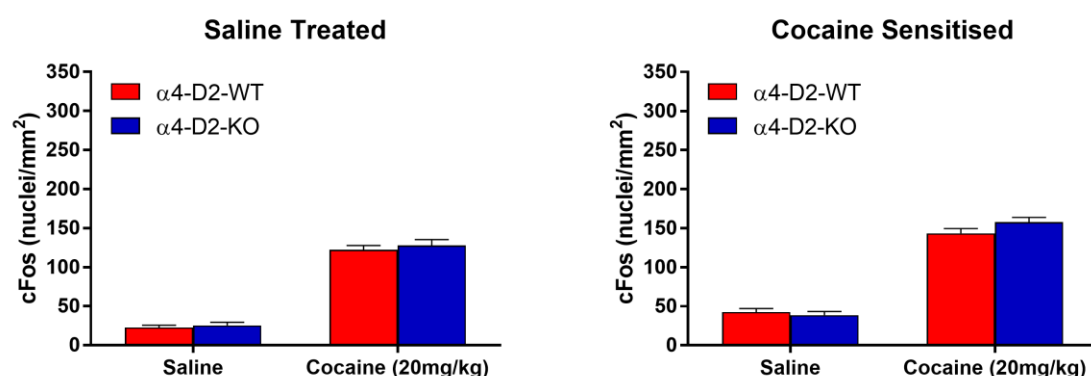
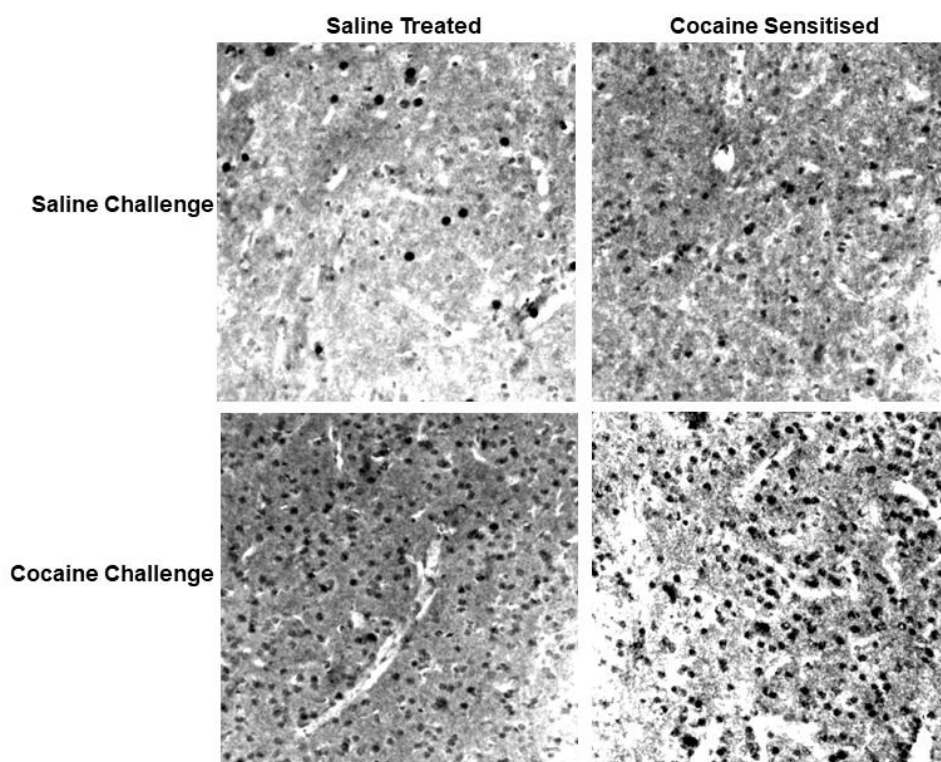


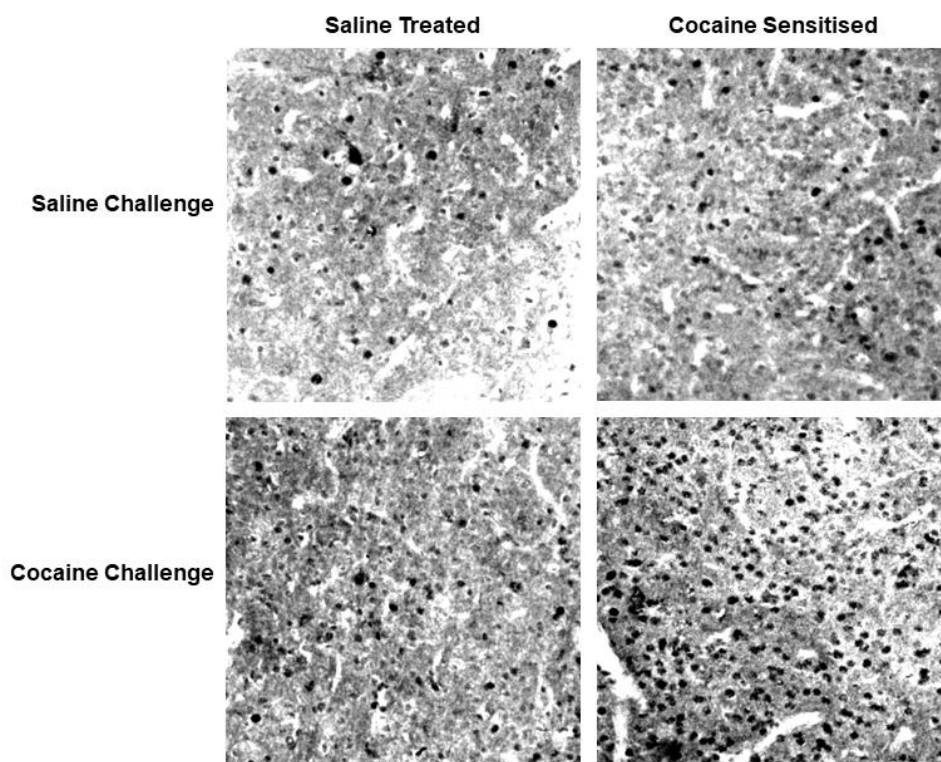
Figure 5.19. Effect of IP saline or cocaine (20mg/kg) injection on cFos expression the NAc Core and Shell of cocaine-sensitised or saline-treated $\alpha 4$ -D2-WT and $\alpha 4$ -D2-KO mice ($n=8$ per group). In saline-treated mice acute cocaine challenge increased cFos expression in the NAc Core ($p<0.001$) and this was not

different between genotypes. In saline-treated mice acute cocaine challenge increased cFos expression in the NAc Shell ($p < 0.001$) and this was not different between genotypes. In cocaine-sensitised mice cocaine challenge increased cFos expression in the NAc Core ($p < 0.001$) and this was not different between genotypes ($p < 0.001$). In cocaine-sensitised mice acute cocaine challenge increased cFos expression in the NAc Shell ($p < 0.001$) and this was not different between genotypes ($p < 0.001$). Cocaine challenge induced greater cFos expression in the NAc Core of cocaine-sensitised than saline-treated mice ($p < 0.01$) and this was not different between genotypes. Cocaine challenge induced greater cFos expression in the NAc shell of cocaine-sensitised than saline-treated mice ($p < 0.01$) and this was not different between genotypes. Cocaine challenge induced greater cFos expression in the Dorsal Striatum of saline-treated ($p < 0.001$) and cocaine-sensitised mice ($p < 0.001$) and this was not different between genotypes. Error bars represent SEM.

**α 4-D2-WT
NAc Core**



**α 4-D2-KO
NAc Core**



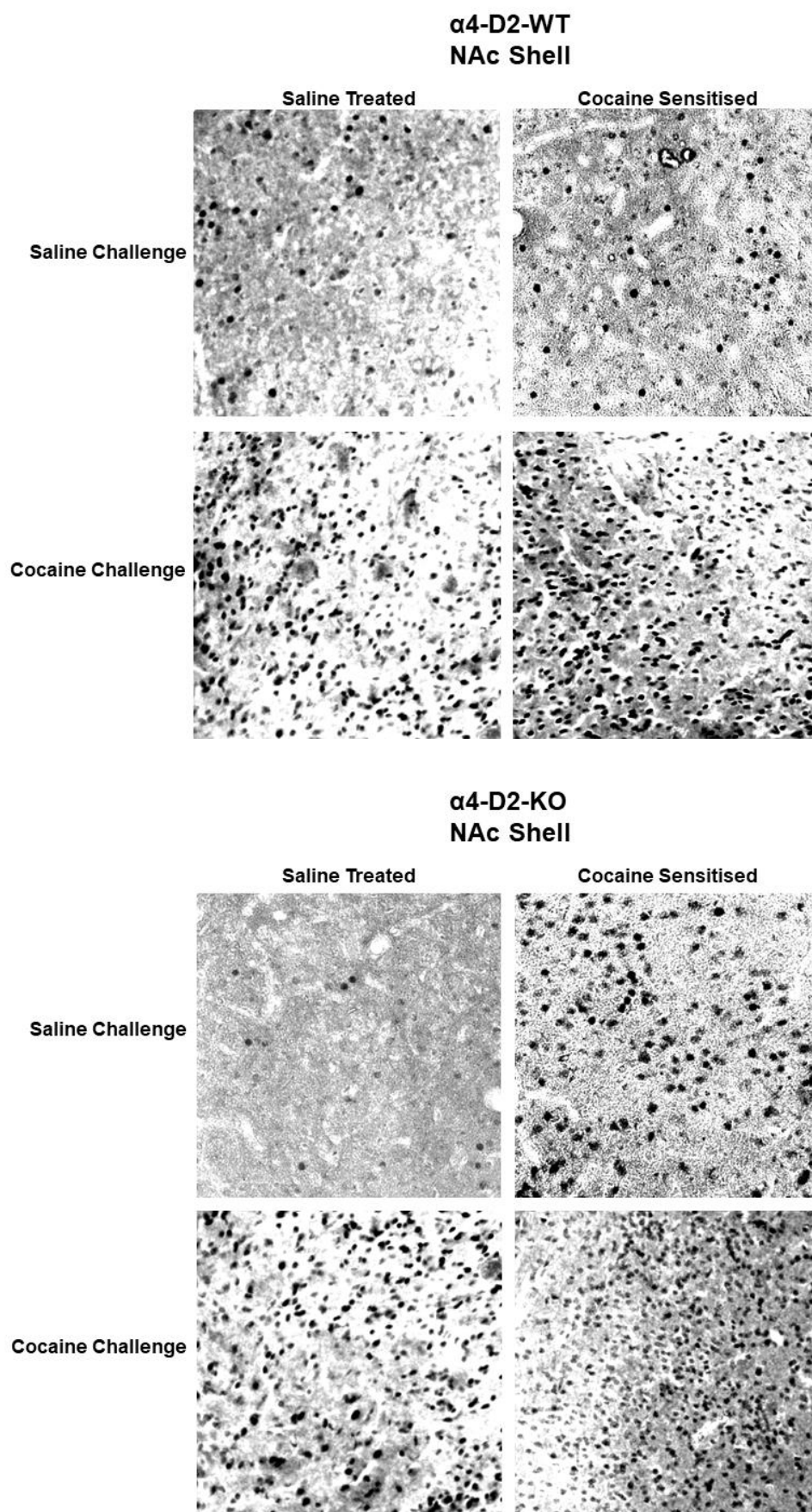


Figure 5.20. Representative images NAC Core and Shell following cocaine (20mg/kg) and saline challenge in cocaine-sensitised and saline-treated α 4-D2-WT and α 4-D2-KO mice.

5.3.14. Behavioural Sensitisation to Cocaine in $\alpha 4$ -KO and $\alpha 4$ -WT mice

Locomotor activity during the initial habituation session (following IP saline injection) revealed no significant differences in baseline locomotor activity between $\alpha 4$ -WT (n=16) and $\alpha 4$ -KO (n=16) mice (Figure 5.21, non-significant effect of genotype $F_{(1,28)} = 0.51$, $p = 0.48$, NS).

Comparison of activity in session 1 revealed that acute-cocaine injection (10mg/kg) significantly increased locomotor activity (Figure 5.21, significant main effect of treatment-drug $F_{(1,28)} = 25.7$, $p < 0.001$), to a similar extent in $\alpha 4$ -KO (n=8) and $\alpha 4$ -WT (n=8) mice (Figure 5.21, non-significant genotype by treatment-drug interaction $F_{(1,28)} = 0.58$, $p = 0.45$, NS).

Repeated, intermittent injections of cocaine, but not saline, induced an increase in locomotor activity over the course of 10 sessions (Figure 5.21, significant main effect of treatment-drug, $F_{(1,28)} = 79.95$, $p < 0.001$; significant session by treatment-drug interaction, $F_{(1,28)} = 6.88$, $p < 0.01$). Saline-treated $\alpha 4$ -WT and $\alpha 4$ -KO mice showed a similar locomotor activity over 10 sessions (Figure 5.21, non-significant session by genotype interaction $F_{(9,25)} = 0.006$, $p = 0.94$, NS). Cocaine-induced activity was similar in both genotypes (Figure 5.21, non-significant session by genotype by treatment-drug interaction, $F_{(9,124)} = 0.12$, $p = 0.74$, NS).

To compare cocaine-sensitisation between genotypes we measured the difference in activity between session 1 and session 10. Comparison of this difference confirmed that both genotypes sensitised to cocaine by a similar magnitude (Figure 5.21, significant effect of treatment-drug, $F_{(1,28)} = 19.22$, $p < 0.001$, non-significant treatment-drug by genotype interaction, $F_{(1,28)} = 0.58$, $p = 0.45$, NS).

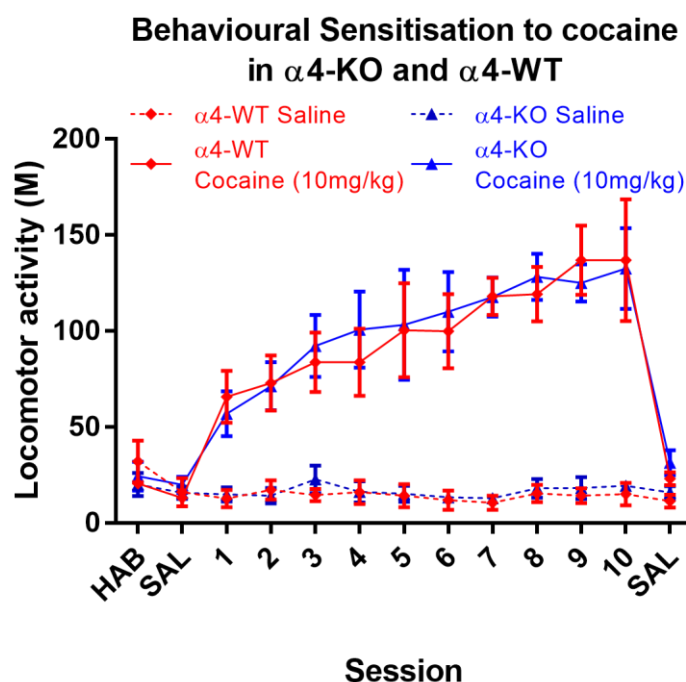


Figure 5.21. Effect of repeated intermittent cocaine on locomotor activity in $\alpha 4$ -WT and $\alpha 4$ -KO mice (per genotype; saline $n=8$, cocaine $n=8$). Locomotor activity was increased over the course of 10 sessions of cocaine administration ($p<0.001$) similarly in both genotypes. In session 1 acute cocaine injection increased locomotor activity similarly in both genotypes. Error bars represent SEM.

5.3.15. Conditioned Activity in $\alpha 4$ -KO vs $\alpha 4$ -WT animals

Following 10 days of repeated cocaine, but not saline, mice showed increases in activity following a saline injection in the cocaine-paired environment (Figure 5.22, significant main effect of treatment-drug, $F_{(1,28)} = 9.63$, $p < 0.001$). Conditioned activity was similar in $\alpha 4$ -KO ($n=8$) and $\alpha 4$ -WT ($n=8$) mice (Figure 5.22, non-significant genotype by treatment-drug interaction $F_{(1,28)} = 0.005$, $p = 0.942$, NS).

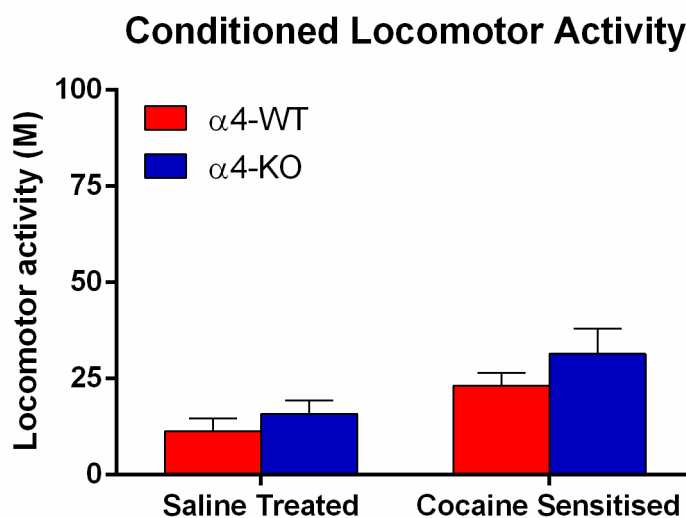


Figure 5.22. Conditioned activity in cocaine-sensitised or saline-treated α4-WT and α4-KO mice (n=8 per group). Locomotor activity following saline administration was significantly increased in cocaine-sensitised mice, compared with saline-treated mice ($p < 0.001$), and this was not different between genotypes. Error bars represent SEM.

5.3.16. Response to 20mg/kg cocaine challenge in sensitised and non-sensitised α4-KO vs α4-WT animals

Compared with saline, cocaine challenge (20mg/kg) significantly increased locomotor activity in saline-treated mice (Figure 5.23, significant main effect of challenge-drug $F_{(1,16)} = 88.4$, $p < 0.001$), and this was not significantly different between in α4-KO (n=4) and α4-WT (n=4) mice (Figure 5.23, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.31$, $p = 0.59$, NS; non-significant main effect of genotype, $F_{(1,16)} = 0.11$, $p = 0.75$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased locomotor activity in cocaine-sensitised mice (Figure 5.23, significant main effect of challenge-drug $F_{(1,16)} = 98.78$, $p < 0.001$), and this was not significantly different between in α4-KO (n=4) and α4-WT (n=4) mice (Figure 5.23, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 0.063$, $p = 0.81$, NS; non-significant main effect of genotype, $F_{(1,16)} = 0.13$, $p = 0.72$, NS).

Cocaine-induced locomotor activity was significantly greater in cocaine-sensitised than non-sensitised mice (Figure 5.23, significant main effect of

treatment-drug $F_{(1,16)} = 19.66$, $p < 0.001$, NS) and this was not significantly different between in $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice (Figure 5.23, non-significant treatment-drug by genotype interaction $F_{(1,16)} = 0.27$, $p = 0.61$, NS; non-significant main effect of genotype, $F_{(1,16)} = 0.001$, $p = 0.99$, NS).

Cocaine Challenge Induced Locomotor Activity

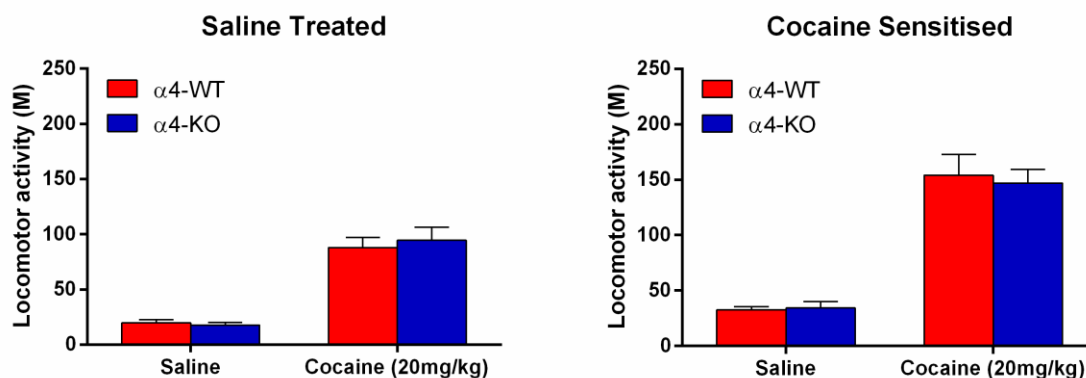


Figure 5.23. Effect of challenge dose of 20mg/kg cocaine on saline-treated and cocaine-sensitised $\alpha 4$ -WT and $\alpha 4$ -KO mice ($n=8$ per group). Cocaine significantly increased locomotor activity ($p<0.001$) to a greater extent in cocaine-sensitised mice than saline-treated mice ($p<0.01$) similarly in both genotypes. Error bars represent SEM.

5.3.17. In Situ Hybridisation to examine cFos in D1/D2 neurons of saline-treated vs cocaine-sensitised $\alpha 4$ -WT and $\alpha 4$ -KO mice

To analyse the effects of acute cocaine in sensitised and non-sensitised animals we compared cFos expression in D1 and D2 neurons in the NAc Core and Shell following an acute challenge of either saline or cocaine (20mg/kg) in cocaine-sensitised vs saline-treated animals.

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc Core of saline-treated mice (Figure 5.24, significant main effect of challenge-drug $F_{(1,16)} = 200.01$, $p<0.001$) to a greater extent in $\alpha 4$ -KO ($n=4$) than $\alpha 4$ -WT ($n=4$) mice (Figure 5.24, significant challenge-drug by genotype interaction, $F_{(1,16)} = 5.72$, $p < 0.05$). There was no significant difference in cocaine induced cFos between D1 and D2 neurons in the NAc core

(Figure 5.24, non-significant neuron-type by challenge drug interaction, $F_{(1,16)} = 0.68$, $p = 0.68$, NS) and this was not significantly different between $\alpha 4$ -KO and $\alpha 4$ -WT mice (Figure 5.24, non-significant neuron-type by genotype by challenge drug interaction, $F_{(1,10)} = 0.015$, $p = 0.9$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc Core of cocaine-sensitised mice (Figure 5.24, significant main effect of challenge-drug $F_{(1,16)} = 63.05$, $p < 0.001$). Cocaine-induced cFos was greater in D1 than D2 neurons in the cocaine-sensitised NAc Core (Figure 5.24, significant neuron-type by challenge-drug interaction, $F_{(1,10)} = 14.64$, $p < 0.01$), and this was increased in $\alpha 4$ -KO ($n=4$) compared with $\alpha 4$ -WT ($n=4$) mice (Figure 5.24, significant neuron-type by challenge-drug by genotype interaction, $F_{(1,16)} = 18.73$, $p < 0.001$).

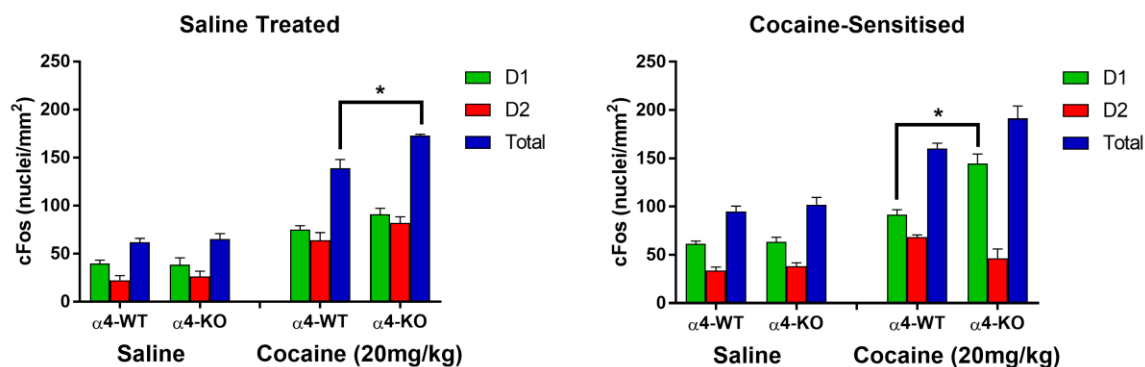
Cocaine-induced cFos in the NAc core was significantly greater in cocaine-sensitised than saline-treated mice (Figure 5.24, significant main effect of treatment-drug $F_{(1,16)} = 5.39$, $p < 0.05$) and this was not significantly different between $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice (Figure 5.24, non-significant treatment-drug by genotype interaction $F_{(1,16)} = 0.031$, $p = 0.86$, NS). Cocaine-induced cFos was increased to a greater extent in D1 neurons of cocaine-sensitised than saline-treated mice (Figure 5.24, significant neuron-type by treatment-drug interaction, $F_{(1,16)} = 21.85$, $p < 0.001$) and this effect was greater in $\alpha 4$ -KO mice (Figure 5.24, significant neuron-type by treatment-drug by genotype interaction, $F_{(1,16)} = 12.87$, $p < 0.01$).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc shell of saline-treated mice (Figure 5.24, significant main effect of challenge-drug $F_{(1,16)} = 74.05$, $p < 0.001$) and this was not significantly different between $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice (Figure 5.24, non-significant challenge-drug by genotype interaction, $F_{(1,16)} = 2.83$, $p = 0.12$, NS). There was no significant difference in cocaine induced cFos between D1 and D2 neurons in the NAc shell of saline-treated mice (Figure 5.24, non-significant neuron-type by challenge drug interaction, $F_{(1,16)} = 0.82$, $p = 0.38$, NS) and this was not significantly different between $\alpha 4$ -KO and $\alpha 4$ -WT mice (Figure 5.24, non-significant neuron-type by genotype by challenge drug interaction, $F_{(1,10)} = 1.21$, $p = 0.3$, NS).

Compared with saline, cocaine challenge (20mg/kg) significantly increased cFos expression in the NAc shell of cocaine-sensitised mice (Figure 5.24, significant main effect of challenge-drug $F_{(1,16)} = 220.04$, $p < 0.001$) to a greater extent in $\alpha 4$ -KO than $\alpha 4$ -WT mice (Figure 5.24, significant challenge-drug by genotype interaction, $F_{(1,10)} = 7.43$, $p < 0.05$). Cocaine-induced cFos was greater in D1 than D2 neurons in the cocaine-sensitised NAc shell (Figure 5.24, significant neuron-type by challenge-drug interaction, $F_{(1,10)} = 29.65$, $p < 0.001$), and this was not significantly different between $\alpha 4$ -KO ($n=4$) and $\alpha 4$ -WT ($n=4$) mice (Figure 5.24, non-significant neuron-type by challenge-drug by genotype interaction, $F_{(1,16)} = 0.001$, $p = 0.99$).

Cocaine-induced cFos in the NAc shell was significantly greater in cocaine-sensitised than saline-treated mice (Figure 5.24, significant main effect of treatment-drug $F_{(1,16)} = 73.38$, $p < 0.001$) to a greater extent in $\alpha 4$ -KO ($n=4$) than $\alpha 4$ -WT ($n=4$) mice (Figure 5.24, significant treatment-drug by genotype interaction $F_{(1,16)} = 9.89$, $p < 0.01$, NS). Cocaine-induced cFos was increased to a greater extent in D1 neurons of cocaine-sensitised than saline-treated mice (Figure 5.24, significant neuron-type by treatment-drug interaction, $F_{(1,16)} = 13.7$, $p < 0.001$), and this was not significantly different between $\alpha 4$ -KO and $\alpha 4$ -WT mice (Figure 5.24, non-significant neuron-type by treatment-drug by genotype interaction, $F_{(1,16)} = 2.09$, $p = 0.12$, NS).

Cocaine-induced cFos in NAc Core



Cocaine-induced cFos in NAc Shell

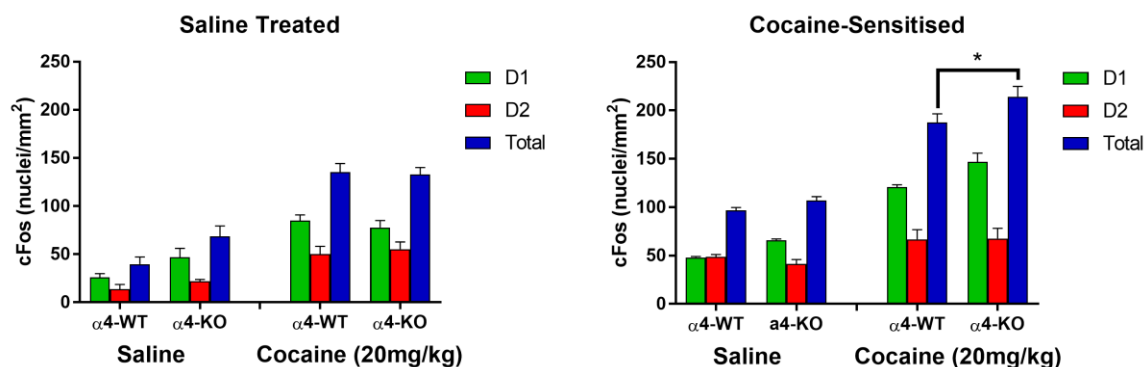
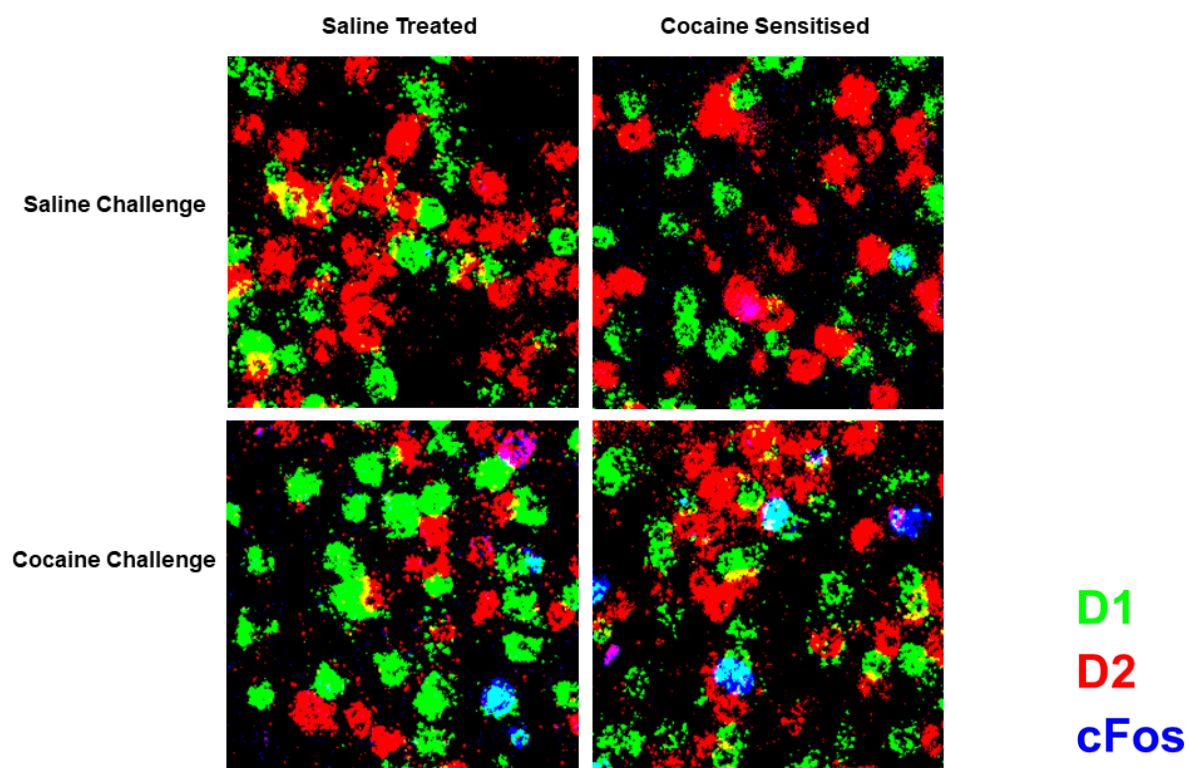


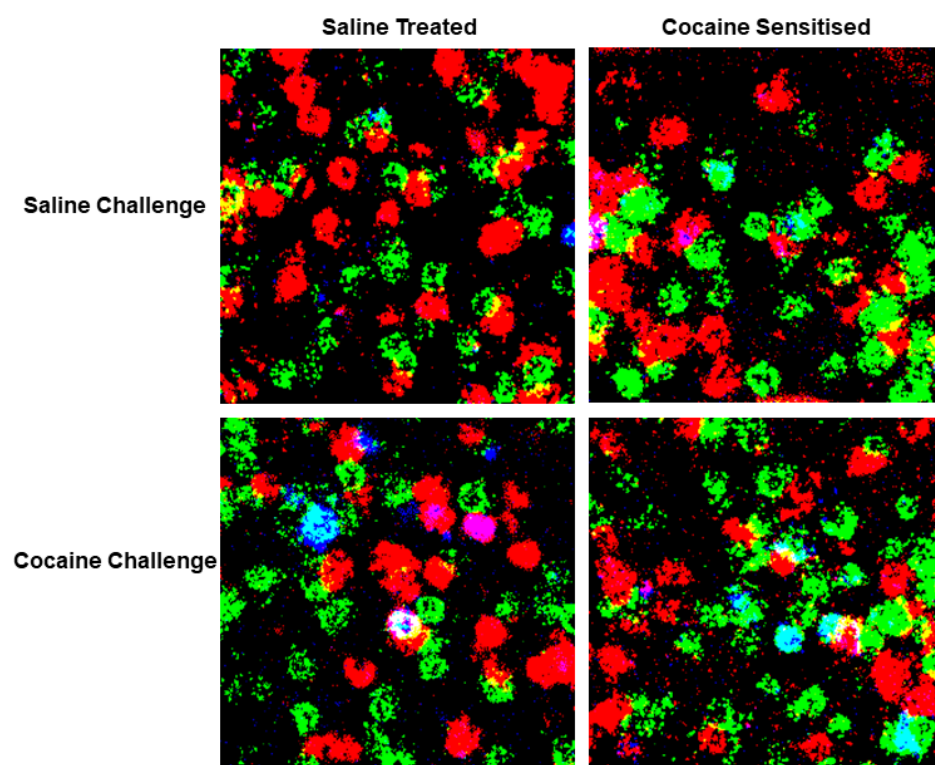
Figure 5.24. Effect of IP saline or cocaine (20mg/kg) injection on cFos expression the NAc Core and Shell of cocaine-sensitised or saline-treated α4-D1-WT and α4-D1-KO mice (n=8 per group). In saline-treated mice acute cocaine challenge increased cFos expression in D1 and D2 neurons in the NAc Core ($p < 0.001$) and this was increased in α4-KO mice ($p < 0.05^*$). In saline-treated mice acute cocaine challenge increased cFos expression in D1 and D2 neurons in the NAc Shell ($p < 0.001$) and this was not different between genotypes. In cocaine-sensitised mice acute cocaine challenge increased cFos expression, preferentially in D1 neurons, in the NAc Core ($p < 0.01$) and this was increased in α4-KO mice ($p < 0.001^*$). In cocaine-sensitised mice acute cocaine challenge increased cFos expression in the NAc Shell, preferentially in D1 neurons ($p < 0.001$) and cFos expression was increased in α4-KO mice ($p < 0.001$). Cocaine challenge induced greater cFos expression in the NAc Core of cocaine-sensitised than saline-treated mice, preferentially in D1 neurons ($p < 0.001$) and this was

increased in $\alpha 4$ -KO mice ($p < 0.01$). Cocaine challenge induced greater cFos expression in the NAc shell of cocaine-sensitised than saline-treated mice, to a greater extent in $\alpha 4$ -KO mice ($p < 0.01^*$) and preferentially in D1 neurons ($p < 0.001$) which was not different between genotypes. Error bars represent SEM.

**α 4-WT
NAc Core**



**α 4-KO
NAc Core**



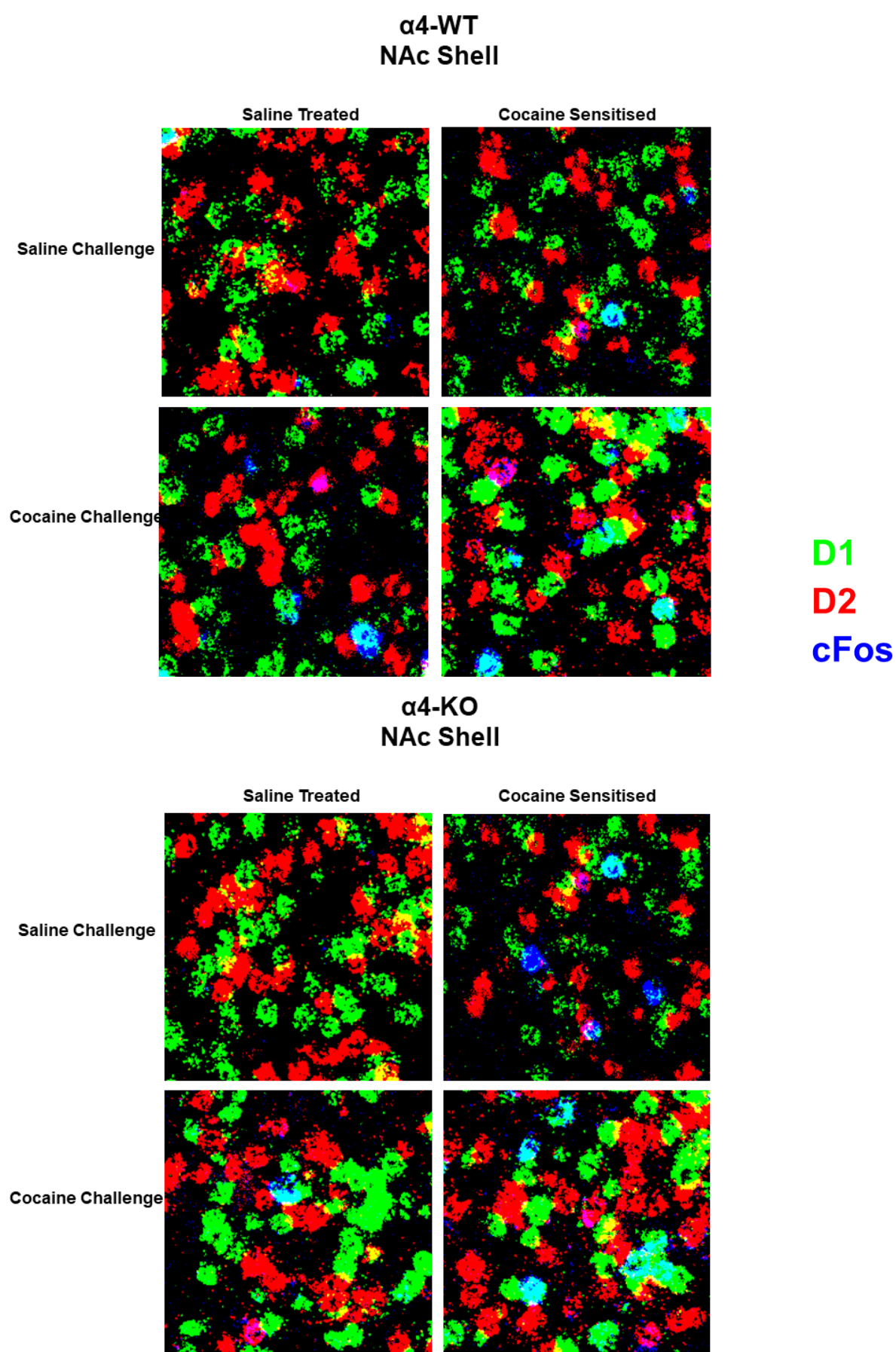


Figure 5.25. Representative images NAc Core and Shell following cocaine (20mg/kg) and saline challenge in cocaine-sensitised and saline-treated α 4-WT and α 4-KO mice. Green = D1, Red = D2, Blue = cFos.

5.4. Discussion

To investigate whether acute or chronic cocaine had different effects on NAc neuronal populations depending on $\alpha 4$ -GABA_AR expression we examined cFos expression following acute cocaine administration in naïve or cocaine-sensitised $\alpha 4$ -KO and $\alpha 4$ -WT mice. We replicated previous findings that mice with a constitutive deletion of GABA_AR $\alpha 4$ -subunit do not show a significant difference from their wildtype counterparts in their locomotor response to acute cocaine at various doses. Furthermore, we confirmed that repeated intermittent cocaine was able to dose-dependently increase locomotor activity equally in both wildtype and GABA_AR $\alpha 4$ -subunit knockout mice (Macpherson, 2013).

However, despite a lack of behavioural deficits in $\alpha 4$ KO mice we identified several differences in cocaine-related cFos expression compared to wild type mice. Acute cocaine administration induced greater cFos expression in the NAc core of naïve $\alpha 4$ -KO mice than $\alpha 4$ -WT controls whilst cocaine induced cFos in the NAc shell was similar in both genotypes. These differences are only present following cocaine administration indicating that $\alpha 4$ -GABA_ARs are modulating neuron ensembles responsive to both acute cocaine administration.

Subsequently, we found that this pattern was altered by chronic cocaine treatment, whereby cocaine-induced cFos was elevated in both the NAc Core and Shell of cocaine-sensitised $\alpha 4$ -KO mice compared with $\alpha 4$ -WT controls. This suggests that $\alpha 4$ is expressed in neural ensembles within the NAc Shell that increase their cFos expression as animals become sensitised to cocaine. When compared with results from acute administration this represents a transition of cocaine dependant activity from the NAc Core to NAc Shell in $\alpha 4$ -GABA_AR expressing neurons that is due to sensitisation. Cocaine challenge also increased cFos expression in the Dorsal striatum; however, the difference between genotypes was only present in the NAc. This may be due to relative greater expression of $\alpha 4$ -GABA_ARs in the NAc (Pirker *et al.*, 2000; Schwarzer *et al.*, 2001; Wisden *et al.*, 1991).

By combining acute and chronic cocaine manipulations into a single experiment and using a multi-probe fluorescent-in-situ-hybridisation we were further able to identify the neuronal subpopulations involved in this effect. The increased cFos

induced by cocaine, in the NAc Core of naïve $\alpha 4$ -KO mice and both NAc Core and Shell of cocaine-sensitised $\alpha 4$ -KO mice compared with their $\alpha 4$ -WT controls, is localised predominantly in D1 expressing neurons (Fig). Cocaine is known to preferentially induced cFos expression in D1 neurons where it facilitates expression of behavioural sensitisation (Zhang *et al.*, 2006). According, relief of tonic inhibition on D1 neurons appears to facilitate this cFos expression. We may therefore have expected an accompanying increase in behavioural sensitisation which is not present in $\alpha 4$ -KO mice. Our results indicate that there must be a mechanism outside of D1 expressing neurons that is preventing this.

Previously we have suggested that lack of effects on behavioural sensitisation in $\alpha 4$ -KO mice may be due to neural compensatory mechanism such as the upregulation of $\alpha 2$ subunits (Macpherson, 2013). However, electrophysiological evidence indicates that deletion of GABA_ARs $\alpha 4$ -subunits has no impact on the kinetics of the phase currents mediated by synaptic receptors within the NAc (Maguire *et al.*, 2014). Furthermore, our cFos data demonstrate that $\alpha 4$ positively affects cocaine induced activity and signalling in D1 neurons.

We therefore suggest that disinhibition of D2 expressing neurons, either on cholinergic interneurons or MSNs, opposes the behavioural effects of disinhibited D1 MSNs and that if both populations are disinhibited concurrently then any resulting effects may be opposing and cancel each other out. This would be the reverse of the effect observed when deletion of NMDARs in D2 MSNs normalized sensitization in mice already lacking NMDARs in D1 receptors (Beutler *et al.*, 2011).

This hypothesis is supported by our finding that specific deletion of $\alpha 4$ -GABA_ARs in D1 expressing neurons alone had identical effects on patterns of overall cFos expression under the same experimental conditions. Furthermore, this was accompanied by an accelerated acquisition of behavioural sensitisation in $\alpha 4$ -D1-KO mice compared with $\alpha 4$ -D1-WT controls. A further experiment using the multi-probe fluorescent-in-situ-hybridisation method in $\alpha 4$ -D1-KO animals following the same behavioural tests is required to verify that increased cFos in D1 neurons underlies these effects in both genotypes. Conversely, following inactivation of D2-MSNs in the NAc using conditionally expressed Tetanus toxin mice exhibit a delayed acquisition of cocaine-sensitisation (Hikida *et al.*, 2010).

This suggests that either disinhibition of D1 MSNs or inhibition of opposing D2 MSNs results in a preference of behaviours associated with direct pathway activation i.e. cocaine potentiated activity.

Conditional deletion of $\alpha 4$ -GABA_ARs in D1 neurons does not affect baseline locomotor behaviour or cFos expression in the NAc Core or Shell. This may be because although $\alpha 4$ is slightly expressed throughout the striatum it is most highly expressed in the NAc which is known to modulate drug potentiated, rather than more general locomotor behaviour (Costall *et al.*, 1977; Campbell *et al.*, 1997). This is also reflected in similar baseline levels of cFos in $\alpha 4$ -KO and $\alpha 4$ -D1-KO and their wildtype controls, suggesting that $\alpha 4$ -GABA_ARs oppose neural activity induced by cocaine without affecting baseline activity. Accordingly, intra-NAc THIP attenuated cocaine potentiation of behaviours (locomotor activity, CPP and CRf) but not baseline behaviour (Macpherson, 2013).

Previous studies of cocaine-induced adaptation of neuronal spine-density have observed a transition of neuroplasticity from NAc Core to Shell (Marie *et al.*, 2012). The investigators blocked increases in spine density on neurons in the NAc Core by using a protein synthesis inhibitor directly following cocaine injection, which was sufficient to abolish cocaine CPP. Administering the inhibitor in the NAc Shell immediately after cocaine injection had no effect but at 4h after cocaine injection it reduced spine density. Importantly, cocaine-induced spine density in the NAc Shell was blocked by inhibition of the NAc Core indicating that plasticity in the NAc core is essential to induce plasticity in the shell, necessary for cocaine reward. We may therefore suggest that increased cFos plasticity in the NAc core of $\alpha 4$ -KO and $\alpha 4$ -D1-KO mice is subsequently transferred to the NAc Shell during sensitisation.

Compared with wildtype controls $\alpha 4$ -D1-KO mice exhibited increased conditioned activity following a saline injection in the previously cocaine paired environment. Unlike locomotor activity and sensitisation, this was not reflected in cFos expression which was similar in cocaine-sensitised $\alpha 4$ -D1-KO mice and wildtype controls following a saline challenge. It has been demonstrated that cocaine induced cFos is increased following sensitisation only when cocaine was administered in the conditioned environment (Mattson *et al.*, 2007). Accordingly, selective inactivation of these neurons with the 'Duan02 inactivation method'

attenuated cocaine-induced locomotor sensitisation in animals receiving cocaine in the drug-paired but not non-paired environment (Koya *et al.*, 2009). It is therefore possible that the increased cFos in D1 neurons we observe in the NAc Shell of $\alpha 4$ -D1-KO mice reflects this environmental association and underlies both conditioned locomotor activity and accelerated acquisition of cocaine sensitisation. Cocaine CPP experiments have implicated the NAc Shell in mediating learned associations between the effects of the drug and the environment while the core might be involved in pharmacological effects of cocaine (Liao *et al.*, 2000; Sellings, McQuade and Clarke, 2006). This increase in cFos is also present in the NAc Shell of $\alpha 4$ -KO mice, although they do not show enhanced conditioned locomotor activity. Therefore, we may also suggest that D2 expressing neurons oppose conditioned activity which may underlie their opposition to increases in behavioural sensitisation when D1-MSNs are disinhibited.

In contrast to constitutive and D1 specific $\alpha 4$ knockouts, D2- $\alpha 4$ -KO mice appear similar to wildtype controls in both in behaviour and cFos expression. This also suggests that differences in constitutive $\alpha 4$ -KO's are be mediated entirely by increased activation of D1 neurons. This may be expected as previous experiments have demonstrated that cocaine induces cFos in striatal neurons via action at D1 but not D2 receptors, and in fact D2 antagonists increase cFos expression (Robertson *et al.*, 1991). Thus, it is possible that changes in activity occur in D2 neurons which are not reflected in cFos activation. As D2 MSNs appear to oppose behavioural sensitisation (Ferguson *et al.*, 2011) we may have expected disinhibition of D2-MSNs by deletion of $\alpha 4$ -GABA_ARs to attenuate sensitisation to cocaine. Optogenetic stimulation of D2 MSNs had no effect of sensitisation (Lobo *et al.*, 2010) indicating that D2 neuron modulation of sensitisation is one-directional and does not directly oppose sensitisation. These data support a model in which D2 neuron activity counterbalances D1 hyperactivity but does not affect sensitisation directly.

In conclusion our data demonstrate that $\alpha 4$ -GABA_ARs mediate tonic inhibition of D1-MSNs in the NAc which are responsive to cocaine. Removal of this tonic inhibition accelerates acquisition of behavioural sensitisation to cocaine and increased conditioned activity, but only if D2 expressing neurons are not-

concurrently disinhibited. We also observe a transfer of potentiated neuronal activity in disinhibited D1 neurons from the NAc Core to NAc Shell following cocaine sensitisation. This may underlie strengthening of environment-drug associations mediated by the NAc Shell.

Chapter 6

General Discussion**6.1. Introduction**

The data presented within this thesis have investigated roles for $\alpha 4$ -GABA_ARs in mediating binge-like alcohol consumption and behaviours associated with addiction to psychostimulants. We have found that $\alpha 4\beta\delta$ -GABA_ARs in the NAc are necessary for high levels of alcohol consumption observed in DID experiments. In addition, $\alpha 4$ -GABA_ARs on D1 expressing neurons modulate the potentiation of several behaviours by cocaine and cocaine-associated environments. Here we will discuss the physiological mechanisms underlying these findings, and their wider implications.

6.2. Summary of Results**6.2.1. Characterising transgenic-mice and viral-vectors used to manipulate GABA_A $\alpha 4$ receptor subunits**

Genotyping and qRT-PCR analysis of NAc and Dorsal Striatum tissue samples confirmed the absence of GABA_AR $\alpha 4$ -subunit DNA and mRNA expression in $\alpha 4$ -KO mice, and a reduction of approximately 50% in $\alpha 4$ -Het mice when compared to $\alpha 4$ -WT controls. These data confirm previous findings that the cre/loxP cleavage of the intended sequence produced a functional effect, blocking the ability of the *Gabra4* gene to produce intact $\alpha 4$ -subunit mRNA in $\alpha 4$ -KO mice (Chandra *et al.*, 2006).

To visualise the expression pattern of GABA_AR $\alpha 4$ subunits, to enable investigation of colocalisation with other neural markers in subsequent analysis, we used a recently developed fluorescent in-situ-hybridisation method (RNAscope). This method has been useful in other studies of low expressing genes as it amplifies mRNA signal for clear visualisation. Unexpectedly, we appeared to detect signal from the probe targeting the *Gabra4* gene in $\alpha 4$ -KO animals where it should be absent. We therefore suggest that $\alpha 4$ signal observed in $\alpha 4$ -KO mice results from either non-specific binding of the probe or that following deletion of exon-3 in $\alpha 4$ -KO mice there remains a truncated mRNA

transcript which does not lead to expression of functional $\alpha 4$ protein but is able to bind the RNAscope probe. The RNAscope probe targets a 1316 base-pair region surrounding the deleted exon-3 but encompassing upstream and downstream which are not deleting in the $\alpha 4$ -KO mouse. If a truncated mRNA is present it could therefore have a large amount of sequence homology with the $\alpha 4$ probe. Despite this problematic 'background' signal we observed higher levels of $\alpha 4$ mRNA specific signal in $\alpha 4$ -WT mice and are therefore able to distinguish $\alpha 4$ -WT and $\alpha 4$ -KO neurons using this method.

To confirm the D1- and D2-specific knockout of $\alpha 4$ in our conditional knockout $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice we performed an in-situ-hybridisation using probes targeting Cre-recombinase, *Gabra4*, and either *Drd* or *Drd2*. No Cre was detected in 'Floxed'- $\alpha 4$ mice which serve as controls for the conditional $\alpha 4$ knockout mice. In $\alpha 4$ -D1-KO mice Cre was colocalized with D1 but not D2 whereas in $\alpha 4$ -D2-KO mice Cre colocalized with D2 and was negatively correlated with D1. We have therefore confirmed that Cre is correctly expressed in the expected neural populations according to the original driver lines. Overall, Cre expression was higher in the NAc of $\alpha 4$ -D2-KO mice than $\alpha 4$ -D1-KO mice. This is also expected as Cre expression was higher in the D2- than D1- BAC-Cre founder line used to breed our conditional knockouts (Gong *et al.*, 2007). In 'Floxed- $\alpha 4$ ', $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice $\alpha 4$ was colocalized with both D1 and D2.

Our analysis revealed that in 'Floxed'- $\alpha 4$ mice this correlation was equal in D1 and D2 neurons. In contrast, we found that in $\alpha 4$ -D1-KO mice $\alpha 4$ was more strongly correlated with D2 and less with D1, whereas in $\alpha 4$ -D2-KO mice the reverse was true; $\alpha 4$ was more strongly correlated with D1 and less with D2. We therefore conclude that the presence $\alpha 4$ probe signal in both populations is due to the background signal (discussed above) and populations of D1 and D2 neurons not expressing Cre in $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice respectively. Thus, converging data evidences a reduction of the $\alpha 4$ mRNA specific signal in D1 or D2 neurons of $\alpha 4$ -D1-KO and $\alpha 4$ -D2-KO mice respectively.

In addition, we used an AAV viral vector to deliver Cre to the NAc of 'Floxed'- $\alpha 4$ mice and demonstrated its ability to reduce $\alpha 4$ subunit mRNA. Expression of the AAV-Cre virus in the NAc was confirmed by immunohistochemistry for the

mCherry marker. We were able to visualise mCherry expression within the targeted region of the NAc core to confirm localisation of the virus. Further qRT-PCR analysis demonstrated substantial knockdown (~60%) of GABA_AR $\alpha 4$ subunit mRNA in the NAc but not in a nearby control region (Dorsal Striatum) when compared with the control AAV-GFP virus or untreated mice. Importantly, we also demonstrated similar levels of $\alpha 4$ expression in 'Floxed'- $\alpha 4$ animals compared with $\alpha 4$ -WT mice which verifies them as equally ecologically representative controls in experiments using manipulations of $\alpha 4$.

The presented data, in addition to previous studies, demonstrate that the GABA_AR $\alpha 4$ subunit is functionally deleted in $\alpha 4$ -KO mice. Further, in D1- and D2- specific conditional knockout mice Cre/loxp deletion has reduced $\alpha 4$ expression in D1 or D2 expressing neural populations respectively. We have also produced an AAV-Cre virus which can be used to locally knockdown $\alpha 4$ expression when surgically injected into a region of interest, the NAc.

6.2.2. The role of $\alpha 4$ -GABA_ARs in mediating binge-like alcohol drinking (Drinking in the Dark)

We have demonstrated a role of $\alpha 4$ -GABA_ARs in mediating binge-like alcohol consumption in mice during 'Drinking in the Dark' experiments. Our data indicate that $\alpha 4$ -GABA_ARs are necessary for the high level of alcohol consumption seen in C57-BL/6J mice as constitutive deletion of $\alpha 4$ was sufficient to reduce alcohol consumption in DID. We have further anatomically refined this result by demonstrating that reducing $\alpha 4$ expression specifically within the NAc is sufficient to reduce binge-like alcohol drinking. Our results agree with multiple studies in which RNAi mediated downregulation of $\alpha 4$ in the NAc reduced ethanol consumption by rats during intermittent access in tests of 'Two-bottle choice' or operant self-administration (Rewal *et al.*, 2009, 2012). At present our results do not suggest $\alpha 4$ -GABA_AR modulation of binge-like alcohol consumption is D1 or D2 dependent and indicate that a combined reduction of $\alpha 4$ -GABA_ARs on D1 and D2 neurons is required to reduce drinking (Table 6.1).

Despite this we were unable to demonstrate robust pharmacological manipulations of drinking via selective $\alpha 4$ -GABA_AR super-agonists. Systemic THIP injections did not reduce ethanol consumption, except at doses which also

reduced water consumption due to sedation. Similarly, we were unable to reduce ethanol drinking by delivering THIP directly to the NAc. This avoids the sedative effect of THIP as intra-NAc THIP at the same dose did not affect baseline locomotor activity (Macpherson, 2013). The highly selective $\alpha 4$ -GABA_AR receptor agonist DS2 also failed to have a significant effect on drinking when delivered to the NAc.

Our experiments identify $\alpha 4$ -GABA_ARs as a potential therapeutic target for treatments of alcoholism. We propose that if partial antagonists with high specificity for $\alpha 4$ -GABA_AR receptors are developed they should be tested in further pre-clinical studies of ethanol consumption such as Drinking in the Dark.

6.2.3. The role of $\alpha 4$ -GABA_ARs in locomotor behaviour and its potentiation by cocaine

The locomotor experiments in this thesis discovered that conditionally deleting $\alpha 4$ -GABA_ARs, thereby relieving tonic inhibition, on D1 MSNs facilitates cocaine-potentiation of locomotor activity at various doses (significantly at 10, 20 and 30 mg/kg). This agrees with multiple studies in which genetic or pharmacological methods to activate D1 MSNs increased cocaine-induced locomotion (Schindler and Carmona, 2002; Bachtell *et al.*, 2005b; A. V Kravitz *et al.*, 2010). We did not observe any differences in baseline locomotor activity following this manipulation, suggesting that $\alpha 4$ -GABA_ARs on D1 MSNs specifically modulate the locomotor activating effects of psychostimulants but not normal initiation of locomotor activity (Table 6.1).

Further, we identified a dissociation between the effects of $\alpha 4\beta\delta$ receptors on D1 and D2 MSNs on cocaine-induced locomotor activity since deletion of $\alpha 4$ in D2 MSNs had no such effect. Based on previous studies using injections of D2 agonists in the NAc we may also have expected an increase in cocaine potentiated locomotor activity (Bachtell *et al.*, 2005b) however those effects were smaller than those produced by D1 agonists therefore deletion of $\alpha 4$ -GABA_ARs may not have a sufficiently strong effect on D2 MSNs to replicate such findings.

Previously, deletion of either GABA_AR $\alpha 4$ -subunits or δ -subunits, often paired in extrasynaptic $\alpha 4\beta\delta$ GABA_ARs, had no influence on baseline locomotion (Herd *et*

al., 2009; Macpherson, 2013) and constitutive GABA_AR $\alpha 4$ -subunit knockout mice showed no difference from wildtype mice in locomotor activity following various doses of cocaine. There is however evidence that when pharmacologically activated $\alpha 4\beta\delta$ receptors oppose the locomotor stimulating effects of cocaine as intra-NAc injections of the agonist THIP attenuated cocaine-induced locomotor activity and this effect was abolished in $\alpha 4$ -KO (Macpherson, 2013). This may be due to the relatively high expression of $\alpha 4$ in the NAc, relative to the rest of the striatum, which is known to mediate the locomotor activating properties of cocaine (Costall *et al.*, 1977; Robinson and Berridge, 1993; Campbell *et al.*, 1997; Schwarzer *et al.*, 2001).

These data suggest that $\alpha 4$ -GABA_ARs in the NAc do not modulate baseline locomotion, but their activation is able to attenuate cocaine-potentiated locomotor activity. Our results indicate that this is most likely mediated by activation of $\alpha 4$ -GABA_ARs on D1 MSNs. This could be fully confirmed by intra-NAc administration of THIP to D1/D2 $\alpha 4$ conditional knockout mice in larger cocaine-dose-response study of locomotor activity. We hypothesise that intra-NAc THIP would not attenuate cocaine-induced locomotor activity in $\alpha 4$ -D1-KO mice, but would in WT or $\alpha 4$ -D2-KO mice.

6.2.4. The role of $\alpha 4$ -GABA_ARs in instrumental responding and its potentiation by cocaine

We have also demonstrated that conditionally deleting $\alpha 4$ -GABA_A receptors on D1 MSNs facilitates cocaine-potentiation of instrumental responding for natural rewards under a PR schedule (Table 6.1). Again, we did not observe any differences in baseline instrumental responding following this manipulation. Whilst $\alpha 4$ -D1-KO show greater enhancement of PR breakpoints following 10mg/kg cocaine they are more severely impaired following 30mg/kg indicating that they are more prone to stereotypy at high doses due to hypersensitivity to the stimulant effects of cocaine. In contrast, deletion of $\alpha 4$ in D2 MSNs had no such effect on cocaine-potentiation of instrumental responding while it similarly had no effect on baseline responding. This contrasts with previous studies where activation of D1 or D2 MSNs systemically or in the NAc facilitated responding under PR schedules reinforced by food or sucrose (Aberman, Ward and Salamone, 1998; Barbano, Le Saux and Cador, 2009). This implies that $\alpha 4$ -

GABA_ARs on MSNs are a oppose effects of psychostimulants but do not alter behaviour under normal conditions.

This dissociated pattern of effects in D1 and D2 specific $\alpha 4$ knockout mice is strikingly similar to those we observed in tests of locomotor activity (outlined above, Table 6.1). These results suggest that $\alpha 4$ -D1-KO mice exhibit 'general increase in approach-investigation' behaviour which is the common underlying mechanism of cocaine-potentiated behaviour in both tests (Ikemoto and Panksepp, 1999). We conclude that increased cocaine potentiation of PR responding in $\alpha 4$ -D1-KO mice is likely due to the enhanced locomotor stimulant effects of cocaine.

We have also identified a behavioural dissociation in the pattern of effects displayed by D1 specific and D2 specific or constitutive $\alpha 4$ knockout mice in tests of locomotor activity/instrumental responding experiments when compared with tests of Conditioned reinforcement (CRf). $\alpha 4$ -D1-KO mice show facilitation of locomotor activity and instrumental responding which was absent in constitutive $\alpha 4$ -KO or $\alpha 4$ -D2-KO mice. Conversely, $\alpha 4$ -KO or $\alpha 4$ -D2-KO mice show enhancement baseline and cocaine potentiated CRf which is absent in $\alpha 4$ -D1-KO mice (Macpherson, 2013). This supports the hypothesis that removal of tonic inhibition of D2 MSNs enhances efficacy of secondary reinforcers and further enhances cocaine's potentiation of CRf independently of its locomotor activating properties or of effects on primary reward value.

6.2.5. The role of $\alpha 4$ -GABA_ARs on D1 and D2 expressing neurons in behavioural sensitisation to cocaine

To investigate whether acute or chronic cocaine had different effects on NAc neuronal populations depending on $\alpha 4$ -GABA_AR expression we examined cFos expression following acute cocaine administration in naïve or cocaine-sensitised $\alpha 4$ -KO and $\alpha 4$ -WT mice. To investigate the role of $\alpha 4$ -GABA_ARs on either D1 or D2 expressing neurons we used D1/D2 specific $\alpha 4$ knockout mice in a standard behavioural sensitisation experiment and performed similar analysis of cFos expression.

We replicated previous findings that mice with a constitutive deletion of GABA_AR $\alpha 4$ -subunit do not show a significant difference from their wildtype counterparts

in their locomotor response to acute cocaine at various doses. Furthermore, we confirmed that repeated intermittent cocaine was able to dose-dependently increase locomotor activity equally in both wildtype and GABA_AR $\alpha 4$ -subunit knockout mice (Macpherson, 2013). However, despite a lack of behavioural deficits in $\alpha 4$ KO mice we identified several differences in cocaine-related cFos expression compared to wild type mice. Acute cocaine administration induced greater cFos expression in the NAc core of naïve $\alpha 4$ -KO mice than $\alpha 4$ -WT controls whilst cocaine induced cFos in the NAc shell was similar in both genotypes. Subsequently, we found that this pattern was altered by chronic cocaine treatment, whereby cocaine-induced cFos was elevated in both the NAc Core and Shell of cocaine sensitised $\alpha 4$ -KO mice compared with $\alpha 4$ -WT controls (Table 6.1).

This suggests that $\alpha 4$ is expressed in neural ensembles within the NAc Shell that increase their cFos expression as animals become sensitised to cocaine. When compared with results from acute administration this represents a transition of cocaine dependent activity from the NAc Core to NAc Shell in $\alpha 4$ -GABA_AR expressing neurons that is due to sensitisation. Further analysis of cFos expression in cocaine sensitised vs non-sensitised $\alpha 4$ -WT and $\alpha 4$ -KO mice revealed that these effects were predominantly mediated by expression of cFos in D1 MSNs in the NAc.

Conditional deletion of $\alpha 4$ -GABA_ARs in D1 neurons did not affect baseline locomotor behaviour or cFos expression in the NAc Core or Shell. This may be because although $\alpha 4$ is slightly expressed throughout the striatum it is most highly expressed in the NAc which is known to modulate drug potentiated, rather than more general, locomotor behaviour (Costall *et al.*, 1977; Campbell *et al.*, 1997). Deletion of $\alpha 4$ -GABA_ARs in D1 neurons resulted in an increased acute response to cocaine and more rapid behavioural sensitisation to cocaine. The $\alpha 4$ -D1-KO mice showed increased locomotor activity in early sessions although they sensitised to a similar endpoint as $\alpha 4$ -D1-WT controls. Furthermore, specific deletion of $\alpha 4$ -GABA_ARs in D1 expressing neurons alone had identical effects on patterns of overall cFos expression under the same experimental conditions (Table 6.1).

In contrast, deletion of $\alpha 4$ -GABA_ARs in D2 expressing neurons had no effect on behavioural sensitisation. This may be expected as previous experiments have demonstrated that cocaine induces cFos in striatal neurons via action at D1 but not D2 receptors, and in fact D2 antagonists increase cFos expression (Robertson *et al.*, 1991). These data support a model in which D2 neuron activity counterbalances D1 hyperactivity but does not affect sensitisation directly.

Compared with wildtype controls $\alpha 4$ -D1-KO mice also exhibited increased conditioned activity following a saline injection in the previously cocaine-paired environment. These results indicate that the increased cFos in D1 neurons we observe in the NAc Shell of $\alpha 4$ -D1-KO mice reflects this environmental association and underlies both conditioned locomotor activity and accelerated acquisition of cocaine sensitisation. As this is not behaviourally reflected in the constitutive $\alpha 4$ knockout we suggest that the concurrent disinhibition of D2 expressing neurons opposes conditioned activity.

These data demonstrate that $\alpha 4$ -GABA_ARs mediate tonic inhibition of D1-MSNs in the NAc which are responsive to cocaine. Removal of this tonic inhibition accelerates acquisition of behavioural sensitisation to cocaine and increased conditioned activity, but only if D2 expressing neurons are not-concurrently disinhibited. We also observe a transfer of potentiated neuronal activity in disinhibited D1 neurons from the NAc Core to NAc Shell following cocaine sensitisation. This may underlie strengthening of environment-drug associations mediated by the NAc Shell.

Behaviour	$\alpha 4$-KO	$\alpha 4$-D1-KO	$\alpha 4$-D2-KO
Drinking in the Dark	Reduced	Normal	Normal
Baseline Locomotor Activity	Normal	Normal	Normal
cFos in NAc Core	Normal	Normal	Normal
cFos in NAc Shell	Normal	Normal	Normal
cFos in Dorsal Striatum	Normal	Normal	Normal
Cocaine-induced locomotor activity	Normal	Enhanced	Normal
cFos in NAc Core	Enhanced	Enhanced	Normal
cFos in NAc Shell	Normal	Normal	Normal
cFos in Dorsal Striatum	Normal	Normal	Normal
Behavioural sensitisation to cocaine	Normal	Accelerated	Normal
cFos in NAc Core	Enhanced	Enhanced	Normal
cFos in NAc Shell	Enhanced	Enhanced	Normal
cFos in Dorsal Striatum	Normal	Normal	Normal
+THIP	Not Blocked	?	?
Fixed Ratio responding	Normal	Normal	Normal
Progressive Ratio responding	Normal	Normal	Normal
Cocaine potentiation of Progressive Ratio responding	Enhanced	Normal	Normal
Cocaine-CPP (MacPherson, 2013)	Normal	Enhanced	Normal
Cocaine-enhanced Cocaine-CPP + THIP (MacPherson, 2013)	Normal Not Blocked	Normal Not Blocked	Absent -
CRf (MacPherson, 2013)	Enhanced	Normal	Enhanced
Cocaine potentiated CRf + THIP (MacPherson, 2013)	Normal Not Blocked	Normal Blocked	Normal Not Blocked

Table 6.1. Summary of the consequences of constitutive or dopamine D1-/D2-expressing neuron specific GABA_AR $\alpha 4$ -subunit knockout mice, and pharmacological activation of $\alpha 4\beta\delta$ -GABA_ARs by THIP.

6.3. By what mechanism do $\alpha 4$ -GABA_ARs modulate alcohol consumption?

Given the role of $\alpha 4$ -GABA_ARs we have identified in CRf for natural rewards and cocaine-conditioned behaviours we may have speculated that their role in alcohol consumption involves similar reward processing mechanisms. However, effects on CRf, CPP, operant responding, locomotor behaviour and sensitisation were all mediated by $\alpha 4$ -GABA_ARs separately on D1 or D2 neuronal populations (Macpherson, 2013). In contrast knockout of $\alpha 4$ in both cell types, either globally or in the NAc, was required to reduce drinking.

Using DID experiments we are unable to examine the motivational/appetitive aspects of $\alpha 4$ -GABA_ARs role in binge-like drinking. We therefore consider two opposing hypotheses; increased tonic inhibition mediated by $\alpha 4$ -GABA_ARs either reduces the “reward” value of ethanol or it enhances satiation such that mice are sated after drinking less alcohol. As reviewed earlier downregulation of $\alpha 4$ or δ reduced operant ethanol self-administration (Rewal *et al.*, 2012) however there was no difference between genotypes in the first 5 minutes of drinking sessions, following several reinforcers, which indicates that this is a difference in consummatory rather than appetitive behaviour. Conversely, mice carrying a mutation of the $\beta 1$ subunit which potentiates GABA_A receptor activity maintained high rates of responding throughout sessions whilst wild-types slowed their rate of as the session progressed consistent with them satiating on alcohol (Anstee *et al.*, 2013).

Alcohol is likely to activate GABA_ARs via upregulation of neurosteroids (Lambert *et al.*, 2003; Finn *et al.*, 2004). At a moderate dose of 50ng the neurosteroid allopregnanolone delivered intracranially (to the lateral ventricle thereby targeting the whole brain) increased alcohol drinking but not appetitive behaviour in mice (Ford *et al.*, 2007). Notably, the investigators used lickometers to observe that allopregnanolone increased drinking bouts in the first 10 minutes, following which drinking was similar to untreated levels. This is in accordance with our hypothesis that activation of $\alpha 4$ -GABA_ARs by neurosteroids opposes satiation and affects consummatory behaviour rather than the reinforcing effects of alcohol. Together these results suggest that tonic inhibition by extrasynaptic GABA_AR receptors opposes ethanol satiation while disinhibition promotes satiation. Under this model the upregulation of $\alpha 4$ following chronic

ethanol administration (Liang *et al.*, 2007) is likely a maladaptive change contributing to maintained ethanol consumption. This may however be opposed by reduced synaptic GABA transmission in MSNs (Wilcox *et al.*, 2014) acting as a homeostatic mechanism. Whilst we have established $\alpha 4$ -GABA_ARs control consummatory behaviour, further experiments are required to investigate alcohol conditioned behaviours (see section 6.8.).

6.4. Does deletion of $\alpha 4$ -GABA_ARs on D1 neurons facilitate cocaine-potentiation of general exploratory behaviours by disinhibition of D1 MSNs within the NAc?

It has been suggested that potentiated locomotor responses following intra-NAc infusions of dopamine agonists may result from a general facilitation of approach-investigation behaviour which is subsequently directed by environmental conditions (Ikemoto and Panksepp, 1999). In a standard locomotor activity chamber increased NAc dopamine transmission may simply stimulate exploratory locomotor activity, whilst in other situations it can facilitate other approach behaviours such as instrumental responding or conditioned activity in operant tasks (Taylor and Robbins, 1986; Cador, Taylor and Robbins, 1991; Kelley and Delfs, 1991).

Under a proposed 'prepare and select' model D1 neurons are involved in preparing several responses whilst D2 neurons are involved in more specific selection based on biological needs (Keeler, Pretsell and Robbins, 2014). Under this model increasing activity in D1 neurons will increase the likelihood of multiple responses whereas increasing activity in D2 neurons will lead to more selective responses. Deletion of $\alpha 4$ from D1 neurons specifically facilitates cocaine's potentiation of locomotor activity, operant responding and behavioural sensitisation, as well as conditioned locomotor activity and CPP. Thus, it appears that $\alpha 4\beta\delta$ GABA_AR inhibition of D1 neurons play a critical role opposing the general behaviour-enhancing properties of psychostimulants. In contrast, deletion of $\alpha 4$ from D2 neurons did not facilitate such a variety of exploratory behaviours but enhanced CRf which requires selective responses to specific cues.

6.5. Does deletion of $\alpha 4$ -GABA_ARs on D1 neurons facilitate environmental conditioning associated with neural ensembles in the NAc Shell?

It is thought that projections from the hippocampus to the NAc, particularly the Shell sub-region, may provide information about environmental cues associated with drugs (Ferbinteanu and McDonald, 2001; Britt *et al.*, 2012). This suggests that the enhanced cocaine-CPP and conditioned locomotor activity seen in $\alpha 4$ -D1-KO mice may result from increased ability of glutamatergic inputs from the hippocampus to excite D1-MSNs within the NAc in the absence of $\alpha 4$ -mediated inhibition. Accordingly, inactivation of NMDA conductance specifically in D1-MSNs decreased acquisition of cocaine-CPP (Heusner and Palmiter, 2005).

Drug associated environmental contexts have been found to promote reinstatement of drug-seeking following extinction which may contribute to relapse in drug addicts (Crombag *et al.*, 2008). Recently, neural ensembles in the NAc Shell have been implicated in the reinstatement of cocaine seeking by cocaine-paired environments. Re-exposure to a cocaine associated context reinstated cocaine seeking and increased cFos expression to a greater extent in the NAc Shell than Core (Cruz *et al.*, 2014). Importantly, inactivation of those neural ensembles by the Daun02 inactivation method in the NAc Shell, but not Core, prevented neuronal activation and the reinstatement of cocaine seeking by the cocaine-associated environment.

Increased cFos in the NAc Shell was associated with conditioned activity in our behavioural sensitisation experiments. Whilst $\alpha 4$ -D1-KO mice showed increased conditioned activity, cFos levels in their NAc Shell were similar to other genotypes. As yet, we do not know if the balance of D1 vs D2 expressing neuronal activation was similar in these mice however this may be elucidated in further experiments (see section 6.8).

6.6. Implications for Drug Abuse

6.6.1. Compounds acting at $\alpha 4$ -GABA_ARs as a treatment for alcoholism?

Drugs which enhance GABA_AR inhibition have been used to treat alcohol use disorders, primarily during withdrawal. Gamma-Hydroxybutyrate (GHB) enhances inhibition by both GABA_A and GABA_B receptors and has been found to

be effective in treating alcoholism in a clinical trial, most likely as an alcohol replacement preventing withdrawal symptoms (Addolorato *et al.*, 1996). GHB has abuse potential and therefore must be carefully prescribed and monitored. Similarly, benzodiazepines have been suggested as an alcohol substitute and are used during withdrawal detoxification, but continued use is rare as they may enhance risk of relapse (Mayo-Smith, 1997; Zack, Poulos and Woodford, 2006). If $\alpha 4$ -GABA_ARs are the subtype primarily affected by alcohol THIP may be able to have similar effects on withdrawal symptoms with fewer side effects due to their specificity. When administered systemically THIP also has sedative properties and, although we did not find a significant effect of intra-NAC THIP on DID, it has been found to enhance drinking in some pre-clinical studies (Boyle *et al.*, 1993), indicating it likely has similar drawbacks to benzodiazepines for this use. DS2 is unlikely to be of therapeutic use due to its limited solubility and ability to cross the blood brain barrier (Jensen *et al.*, 2013).

Electrophysiological evidence indicates that alcohol activates $\alpha 4$ -GABA_ARs indirectly via upregulated neurosteroids (Lambert *et al.*, 2003; Finn *et al.*, 2004). Agents that block the synthesis or action of neurosteroids reduce alcohol consumption in animals (Simms *et al.*, 2012). Finasteride, which blocks the synthesis of neurosteroids including allopregnanolone, was recently reported to reduce alcohol consumption in humans (Irwig, 2014), although it also caused persistent sexual side-effects. The testing of mifepristone in human subjects with alcohol use disorders is in early stages and clinical trials are not yet published (Swift and Aston, 2015). If discovered/developed, compounds that block interaction between neurosteroids and $\alpha 4$ -GABA_ARs may have similar effects on drinking behaviour without off-target effects associated with the depletion of steroids.

6.6.2. Compounds acting at $\alpha 4$ -GABA_ARs as a treatment for cocaine abuse?

In previous studies, systemic or intra-NAC administration of THIP was not able to reduce cocaine-CPP and CRf responding under drug-free (baseline) test conditions (Macpherson, 2013). However, THIP did reduce cocaine-enhancement of cocaine-CPP, CRf responding and locomotor activity, as well as behavioural sensitisation to cocaine. These results indicate that THIP is able to block the

energising effects of cocaine. The present data suggest this is primarily mediated by $\alpha 4$ -GABA_ARs on D1 neurons and may, in part, result from attenuated drug-environment associations. Thus, THIP has potential therapeutic value in blocking the hyperlocomotor of cocaine and its potentiation of drug-seeking, although it is yet unclear what the effects may be in humans.

Unfortunately, when administered systemically, THIP also has sedative properties and therefore may have limited therapeutic use in treating cocaine abuse. Furthermore, our results implicate $\alpha 4$ -GABA_ARs in promoting alcohol consumption, as do several other studies (Rewal *et al.*, 2009, 2012). Although our data do not demonstrate that THIP or DS2 increase alcohol drinking, published studies did find that THIP increased drinking and we observed trends towards increased drinking following intra-NAc THIP and DS2. We would therefore suggest caution in clinically administering THIP, or other $\alpha 4$ -GABA_AR agonists, especially to poly-drug abusers who drink alcohol.

6.6.3. Investigation of the GABRA4 gene in humans

A linkage and association analysis indicated that the '16-cM' region of chromosome 4p, which contains a cluster of genes encoding GABA_AR subunits such as GABRA2 and GABRA4, was associated with an increased risk of drug dependence (Reich *et al.*, 1998; Edenberg *et al.*, 2004; Enoch, 2008, 2013). Furthermore, single nucleotide polymorphisms (SNPs) of the GABRA2 gene have been associated with alcohol dependence and cocaine addiction in humans (Edenberg *et al.*, 2004; Dixon *et al.*, 2010). In contrast, six GABRA4 SNPs investigated were not significantly correlated with risk for alcohol dependence (Edenberg *et al.*, 2004). Similarly, post-mortem analysis of GABAergic gene expression revealed that the GABRA2 gene was altered in hippocampus of alcohol and cocaine addicts, whereas GABRA4 expression was unaltered (Enoch, 2013).

Given the various effects of $\alpha 4$ -GABA_ARs manipulations on alcohol and cocaine related behaviour described in this thesis we may have expected SNPs or altered expression of the GABRA4 gene in humans to be associated with drug addiction. It is possible that compensatory changes in other genes may mask the behavioural effects of different $\alpha 4$ subunit expression in humans, as has been

observed in $\alpha 4$ -KO mice (Chandra *et al.*, 2006). Based on our data it is possible that only $\alpha 4$ -GABA_ARs within the NAc are involved in alcohol and cocaine related behaviours therefore analysis of tissue from the Striatum and NAc of people with addiction may be more relevant.

6.7. Considerations

6.7.1. The use of THIP/DS2 to target $\alpha 4$ -GABA_ARs

In our DID experiments the GABA_AR agonist THIP was used due to its preferred action at δ -subunit containing GABA_ARs, which within the NAc are largely co-assembled with $\alpha 4$ -subunits in extrasynaptic locations (Pirker *et al.*, 2000; Stephen G. Brickley and Mody, 2012). However, it has been reported that THIP doses over 3 μ M may begin to act at $\gamma 2$ -containing synaptic GABA_ARs in addition to its action at δ -containing extrasynaptic receptors (Ebert *et al.*, 1994; Mortensen *et al.*, 2004, 2010). We therefore also used DS2, a novel positive allosteric modulator of δ -containing GABA_ARs which more specifically targets extrasynaptic $\alpha 4\beta\delta$ receptors. An in-vitro concentration-response study indicated that DS2 produces a similar peak stimulated inhibitory current as THIP in $\alpha 4\beta\delta$ receptors, but, unlike THIP, does not produce any response in $\alpha 4\beta\gamma 2$ or $\alpha 1\beta\gamma 2$ receptors even at high concentrations (up to 10 μ M) (Mortensen *et al.*, 2010; Jensen *et al.*, 2013).

When tested in-vivo systemic administration of DS2 demonstrates a poor brain/plasma ratio, indicating DS2 does not readily cross the blood-brain barrier (Jensen *et al.*, 2013). Accordingly, systemic administration of DS2, even at high doses (up to 100mg/kg), fail to produce the effects in animal models seen with relevant doses of THIP, including reduced locomotor activity and rotarod performance (Wafford and Ebert, 2006; Herd *et al.*, 2009). Furthermore, DS2 is not readily soluble in saline solution. We were able to dilute DS2 in sterile saline containing 2% Tween-20 and 2% DMSO and our results indicate that this buffer did not produce effects on its own when infused intracranially. Based on preliminary studies in WT mice we used a dose of 0.03mM administered intracranially, directly to the NAc (Dixon, Stephens, King, unpublished data).

Immunohistochemical analysis revealed that the expression pattern of the GABA_AR $\alpha 4$ -subunit within the NAc is indistinguishable from that of GABA_AR δ -

subunits, and distinct from expression of the cell adhesion molecule neuroligin2 (NL2), which is selectively expressed in inhibitory synapses (Maguire *et al.*, 2014). Thus, there appear to be few or no synaptic $\alpha 4$ -GABA_ARs within the NAc, indicating that the effects of THIP or DS2 at $\alpha 4$ -GABA_ARs in the NAc are highly likely to be due to an action at extrasynaptic $\alpha 4\beta\delta$ receptors.

6.7.2. cFos

Firstly, because cFos expression is activated by a wide variety of signalling pathways its expression alone cannot provide much information about the mechanisms behind cFos activation in our experiments (Chung, 2015). However, many studies have characterised the mechanisms by which psychostimulants activate cFos activation in MSNs via transmission at D1 dopamine receptors (reviewed, Xu, 2008). This may mean our experiments are insensitive to differences of activity in D2-MSNs.

Secondly, cFos expression does not indicate whether the neurons are activated directly, for example by increased dopamine transmission, or indirectly due to upstream, circuit-level changes. This is particularly problematic when studying effects of systemic drug administration and the basal ganglia where sub-regions receive many different inputs (Robertson *et al.*, 1991).

Third, activity-dependent genes are differentially regulated in different cell types and in distinct brain areas and immediate early gene expression does not always result correlate with neuronal firing (Kawashima, Okuno and Bito, 2014). It is also possible that a stimulus activates a neuron without activating cFos.

Finally, cFos expression is useful for measuring only activation, not inhibition, of neurons. Thus, it can be used to measure disinhibition, as in our sensitisation experiments, but not inhibition mediated by GABA_ARs. This is not necessarily prohibitive to examining the effects of $\alpha 4$ -GABA_AR activation on cFos as THIP or DS2 treated mice could be compared with saline treated controls. Activation of D2Rs by dopamine results in inhibition of neurons, therefore measures of cFos will not provide evidence of increased activation of D2Rs which likely occurs due to increased dopamine release during behavioural sensitisation.

6.7.3. The use of mouse behavioural paradigms to model addiction-associated behaviours

6.7.3.1. Drinking in the Dark

The principal disadvantage of Drinking in the Dark is that no choice is offered. However, a 4-hour period of voluntary fluid deprivation is not sufficient to greatly challenge mice (Lyons *et al.*, 2008), and many genotypes drink very little in DID (Rhodes *et al.*, 2007). The motivation for ethanol consumption in DID is not known, although it is unlikely to be due to calorie seeking and probably not caused by postprandial thirst stemming from feeding (Lyons *et al.*, 2008).

The DID procedure takes advantage of a time-period in the animal's circadian rhythm which is associated with high levels of ingestive behaviour to produce high level of ethanol intake (Rhodes *et al.*, 2005). When C57BL/6J mice were given access to 20% ethanol beginning 3 hours into the *light* cycle consumption of ethanol was and associated BECs were reduced to ~20% relative to 3 hours into the *dark* cycle (Lowery-Gionta *et al.*, 2012). Experiments may be affected by circadian fluctuations in neurophysiological activity, including in neurochemical systems, that have been implicated in binge-like ethanol drinking (Mitchell, Prévot and Beauvillain, 1998; Vidal and Lugo, 2006; Sprow and Thiele, 2012), and yield results that are specific only to that portion of the dark cycle.

In DID experiments mice consume binge-like levels of ethanol during the time of day that they also consume most of their food, therefore it is possible that DID procedures may interfere with normal feeding (or vice versa). This may be more apparent in experiments involving repeated use of DID procedures over days or weeks, as in our intra-NAc drug delivery experiments. Although short-term food restriction did not alter the level of binge-like ethanol drinking with DID procedures (Lyons *et al.*, 2008), it is possible over many sessions this could impact ethanol consumption.

Observations generated with DID procedures in mice may not generalize to other strains or species. Out of a panel of 12 strains tested only C57BL/6J mice achieved BECs above 100 mg/dL (Rhodes *et al.*, 2007). Different ethanol drinking in DID procedures could be attributed to taste reactivity to ethanol. Some strains might develop reduced sensitivity to the aversive taste of ethanol

and thus be willing to consume more. However, C57BL/6J mice show relatively stable ethanol intake over the 4-day DID procedure (Rhodes *et al.*, 2005) and, while repeated episodes of DID promoted significant increases of subsequent continuous 2-bottle-choice ethanol intake, the level of binge-like ethanol drinking did not increase following 40 sessions (Cox *et al.*, 2013).

6.7.3.2. Behavioural Sensitisation

Although neural and behavioural sensitisation is well established in animals it has been questioned whether such sensitisation also occurs in humans. Some studies have reported that repeated intermittent administration of amphetamine resulted in sensitisation of striatal dopamine release (Boileau *et al.*, 2006), whereas others have found that detoxified cocaine addicts exhibit a decrease in methylphenidate or amphetamine evoked dopamine release in the striatum following administration, rather than a sensitised increase (Volkow *et al.*, 1997; Martinez *et al.*, 2007). These studies may be confounded by congenital or otherwise pre-existing differences in dopamine release in people with addiction (Melis, Spiga and Diana, 2005). Similarly, behavioural evidence for progressive drug effects in humans is mixed. Subjects reported increased subjective effects of amphetamine following repeated administration (Strakowski *et al.*, 2001; Boileau *et al.*, 2006), and clinician-rated levels of energy and motor activity were also reported to be increased (Strakowski *et al.*, 1996, 1998). In contrast, other studies found no evidence of increased subjective effects following repeated amphetamine administration (Johanson and Uhlenhuth, 1981; Kelly, Foltin and Fischman, 1991). This may be attributed to the conditions during drug administration in these studies. The expression of sensitisation is modulated by the environmental context of drug administration (Robinson & Berridge, 2008), therefore a drug challenge in the test environment may not result in the expression of behavioural sensitisation as it would in a previously drug-paired context. Further investigation, including environmental manipulations, is required to investigate behavioural sensitisation in humans

6.7.4. Genetic limitations

A major limitation to the use of transgenic mice is that genetic deletion or altered expression of a gene often results in compensatory changes in

expression of other genes and their products. Indeed, previous qRT-PCR analysis of NAc tissue samples revealed that mRNA expression levels of two other GABA_AR subunits were altered following deletion of *Gabra4* either constitutively or specifically or in D1/D2 neurons. Expression of the GABA_AR δ -subunit was reduced, likely due to a lack of $\alpha 4$ -subunits with which δ -subunits would ordinarily co-assemble to form extrasynaptic $\alpha 4$ -GABA_ARs.

In contrast, expression of the GABA_AR $\alpha 2$ -subunit was increased. It is possible that regulation of the *Gabra2* gene was perturbed due to deletion of *Gabra4* as they are located adjacently on chromosome 4. However, electrophysiological evidence demonstrated that deletion of the $\alpha 4$ -subunit did not affect the kinetics of phase currents mediated by synaptic receptors within the NAc (Maguire et al, 2013). This indicates that the increase in $\alpha 2$ -subunit mRNA in the NAc is not translated into $\alpha 2$ -subunit proteins in functional receptors. This could be further confirmed by western blot or immunohistochemical analysis of $\alpha 2$ -subunit protein expression in $\alpha 4$ -KO mice.

Viral vectors have been proposed as a way of altering gene regulation while avoiding compensatory changes as they can be expressed in adult animals (Hommel et al., 2003). However, it is known that GABA_ARs can be rapidly up- or down-regulated under certain physiological conditions, such as following cocaine or alcohol administration (Liang *et al.*, 2007). We used a Cre virus to delete $\alpha 4$ -GABA_ARs specifically within the NAc of 'floxed- $\alpha 4$ mice, which resulted in reduced alcohol consumption in our DID experiments. Whilst we used qRT-PCR analysis to confirm the knockdown we did not analyse the expression of other GABA_ARs. This should be carried out in further experiments to ensure that compensatory changes in other GABA_AR subunits are not the cause of this effect. For example, GABA_AR $\alpha 2$ subunit, which has been implicated in the acute and reinforcing effects of alcohol (Dixon et al., 2012; Edenberg et al., 2004). Furthermore, if any GABA_AR $\alpha 4$ subunit mRNA is differently regulated protein analysis (Western blot or immunohistochemistry) should be used to detect whether this results in changes in expression of functional receptors.

We have previously used qRT-PCR analysis to demonstrate changes in expression of GABA_ARs in the NAc of D1/D2 specific $\alpha 4$ knockout mice, including upregulation of $\alpha 2$ mRNA (Macpherson, 2013). However, expression within the

individual D1/D2 cell populations has not been examined. This could be examined using immunohistochemistry or multi-probe in-situ-hybridisation to colocalise D1/D2 markers with various GABA_AR subunits. These methods are typically less quantitative and, at present, are limited by the number of antibodies/probes that can be co-labelled simultaneously. It may be possible to use Fluorescence-activated cell sorting (FACS) to separate D1 and D2 cell populations for qRT-PCR and Western Blot analysis. Methods have been developed which allow FACS based on immunolabelled cell types (Guez-Barber *et al.*, 2011). This would rely on antibodies for D1/D2 neuron markers which at present show problems with effective application and cross-reactivity in brain tissue. Alternatively, $\alpha 4$ -D1/D2-KO mice could be bred with BAC-mice expressing different fluorochromes under the *Drd1* and *Drd2* promoters, e.g. GFP and td-Tomato as previously demonstrated (Shuen *et al.*, 2008). This would be confounded by additional BAC disruption and expression of fluorochromes although cross-bred, BAC-XFP and WT control mice could be compared to investigate global effects on GABA_AR subunit expression.

6.8. Future Work

6.8.1. Role of $\alpha 4$ in alcohol consumption and reinforcement

In the presented experiments knockdown of $\alpha 4$ in the NAc, using a Cre virus in 'floxed'- $\alpha 4$ mice, was sufficient to reduce drinking under DID conditions. In further experiments we could we do this prior to operant self-administration experiments to investigate the role of $\alpha 4$ -GABA_ARs in motivation for alcohol as well as consumption. If this reduces self-administration, we would replicate findings of Rewal and colleagues (2012) who used RNAi to knockdown $\alpha 4$ in the NAc of rats. Additionally, time-course data obtained in such experiments may indicate whether reduced consumption in $\alpha 4$ -KOs is a result of faster satiation. This would be indicated if levels of drinking are similar early drinking sessions but more rapidly decline (as observed by Rewal *et al.* 2012).

It may be of interest to investigate other regions in which $\alpha 4$ is expressed using similar methods. The thalamus is a good candidate as it is where $\alpha 4$ is most highly expressed (Sur *et al.*, 1999b; Schwarzer *et al.*, 2001) and ethanol is known to potentiate GABAergic tonic inhibition there in wild-type but not $\alpha 4$ -KO

mice (Jia et al. 2007). If depression of the thalamus is necessary to promote ethanol consumption $\alpha 4$ knockdown may result in reduced drinking.

It would be highly useful to examine pharmacological inactivation of $\alpha 4$ -GABA_ARs on alcohol consumption; however, at present there are no antagonists that are specific for $\alpha 4\beta\delta$ receptors. Based on electrophysiological evidence it is likely that alcohol activates $\alpha 4$ -GABA_ARs indirectly via upregulated neurosteroids (Lambert et al., 2003; Finn et al., 2004). We could test this hypothesis in future experiments by investigating whether administration of neurosteroids, for example allopregnanolone, is able to increase alcohol drinking and operant self-administration in $\alpha 4$ -KO mice as it does in WT mice (Janak, et al., 1998; Sinnott et al. 2002).

Finally, to establish whether $\alpha 4$ -GABA_ARs promote alcohol reinforcement it is necessary to use similar genetic manipulations of $\alpha 4$ in ethanol conditioning experiments. Although they often are less robust than when performed using psychostimulants, protocols have been developed to establish responding for ethanol associated cues, ethanol CPP and ethanol sensitisation (Kelley et al., 1997; Phillips et al., 1994; Shahan & Jimenez-Gomez, 2006).

6.8.2. Role of $\alpha 4$ in Cocaine-related behaviours

Following our demonstration that D1 neurons are the primary mediator of cFos differences in cocaine-sensitised $\alpha 4$ -KO it is of immediate interest to conduct similar experiments in D1/D2 specific $\alpha 4$ -GABA_AR knockout mice. We hypothesise that the similar patterns of overall cFos expression observed in $\alpha 4$ -D1-KO mice as in $\alpha 4$ -KO mice are also highly likely to be mediated by D1 neurons following cocaine sensitisation.

Previously we have demonstrated that systemic THIP administration blocks cocaine potentiation of locomotor activity and behavioural sensitisation in WT but not $\alpha 4$ -KO mice (Macpherson, 2013). We could use the same manipulation in D1/D2 specific $\alpha 4$ -GABA_AR knockout mice to identify whether either one or both populations mediates this effect. Outcomes of THIP activating D1 and D2 were dissociated in other behaviours, including cocaine potentiated CPP and CRf (Macpherson, 2013). We hypothesise that THIP would not affect cocaine-potentiated locomotor activity or sensitisation in $\alpha 4$ -D1-KO mice which would

provide secondary confirmation that $\alpha 4$ -GABA_ARs on D1 neurons appear to oppose those behaviours.

As D1 and D2 receptors are widespread throughout the striatum and other regions (Gerfen *et al.*, 1990) it would be useful to provide anatomical specificity to behavioural effects in D1/D2 specific $\alpha 4$ -GABA_AR knockout mice. Previous studies have demonstrated knockdown of $\alpha 4$ -GABA_ARs in the NAc of rats using viral vector mediated RNAi (Rewal *et al.*, 2009). Viral vectors have been developed which conditionally express RNAi sequences in the presence of Cre recombinase (Saunders *et al.*, 2012). This technique could be used in combination with BAC mice expressing Cre under the D1- or D2-promotor to conditionally knockdown Gabra4 in those populations and within specified brain regions such as the NAc. We hypothesise that this would result in similar effects on cocaine-related behaviour observed in our D1/D2 conditional knockout mice due to the established role of the NAc in those behaviours, the high expression of $\alpha 4$ -GABA_ARs there, and its further implication by our cFos analysis of cocaine treated mice.

In our experiments cocaine was continually administered in the cocaine-paired environment during behavioural sensitisation and challenge dosing for cFos analysis. It has been established that cocaine induces greater cFos expression in a cocaine environment (Mattson *et al.*, 2007) and our results suggest that $\alpha 4$ -GABA_ARs on D1 neurons modulate neural ensembles which mediate this environmental effect. It would therefore be of interest to observe the effects of cocaine on cFos expression in a non-cocaine paired environment to see if $\alpha 4$ -GABA_ARs mediated differences are still apparent. If so this would indicate whether $\alpha 4$ -GABA_ARs are directly involved in behavioural sensitisation or that they encode drug-environment association which contributes to increased locomotor activity.

Operant self-administration of drugs is often considered the gold-standard for examining treatments for addiction pre-clinically. It may therefore be of interest to examine constitutive $\alpha 4$ -KO and $\alpha 4$ -D1/D2-KO mice in such experiments. However, the increases in cocaine potentiated locomotor activity and responding for natural rewards observed in $\alpha 4$ -D1-KO mice would likely confound our interpretation of operant self-administration experiments. Drug-associated cues

powerfully drive drug-seeking behaviour (Di Ciano and Everitt, 2004) therefore the role of $\alpha 4$ in conditioned behaviours may be important regardless of its effect on operant self-administration. While we have previously examined the constitutive and D1/D2 specific $\alpha 4$ knockout mice in CRf using food rewards it would be useful to extend these results using cocaine rewarded CRf experiments. This may further indicate whether $\alpha 4$ -GABA_ARs are a useful target to reduce drug seeking following conditioned cues.

6.9. Conclusion

In conclusion $\alpha 4$ -GABA_ARs are modulators of the excitability of NAc MSNs, and therefore play an important role in controlling consummatory, locomotor and conditioned behaviours. Deletion of $\alpha 4$ -GABA_ARs globally or within the NAc is sufficient to dramatically reduce binge-like alcohol drinking. In contrast deletion of $\alpha 4$ -GABA_ARs specifically from D1 expressing neurons is required to facilitate cocaine-potentiation of locomotor activity, operant responding cocaine-sensitisation and conditioned locomotor activity. These behavioural differences are accompanied by increased cFos expression in the NAc, acutely in the Core then in the Shell following sensitisation. These data indicate $\alpha 4$ -GABA_ARs within the NAc play different roles controlling alcohol consummatory behaviour and cocaine conditioned behaviours, both of which are associated with addiction.

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